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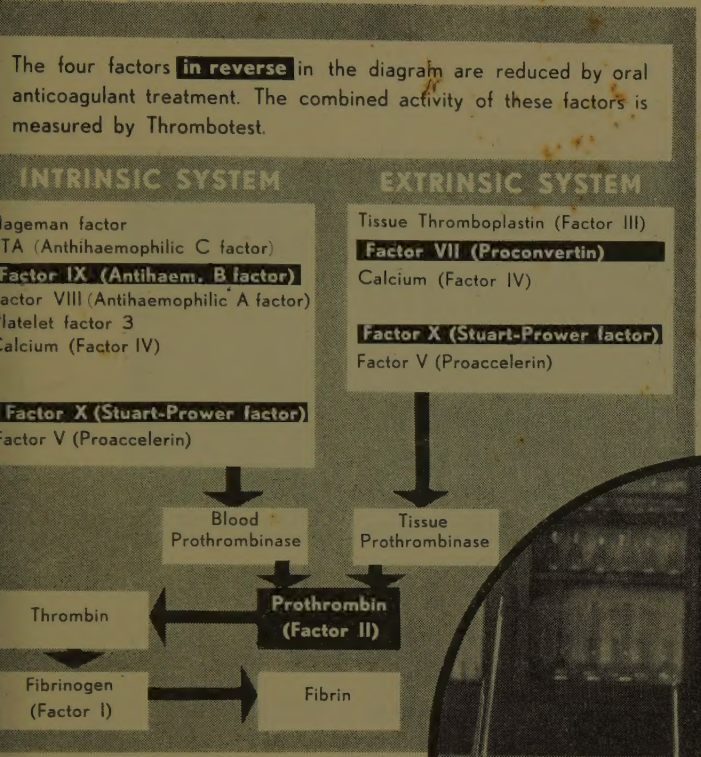

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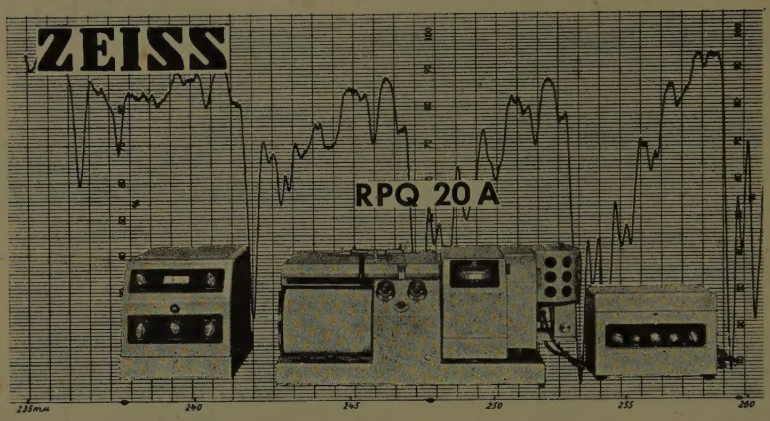

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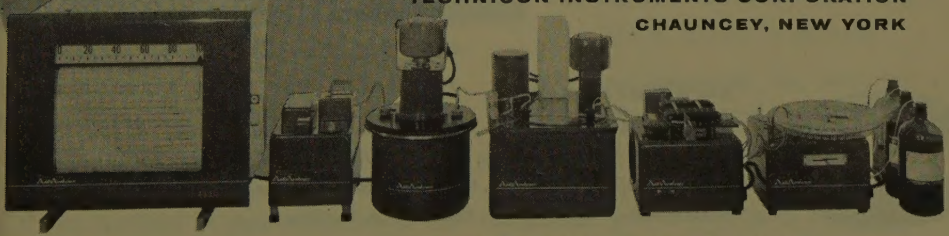

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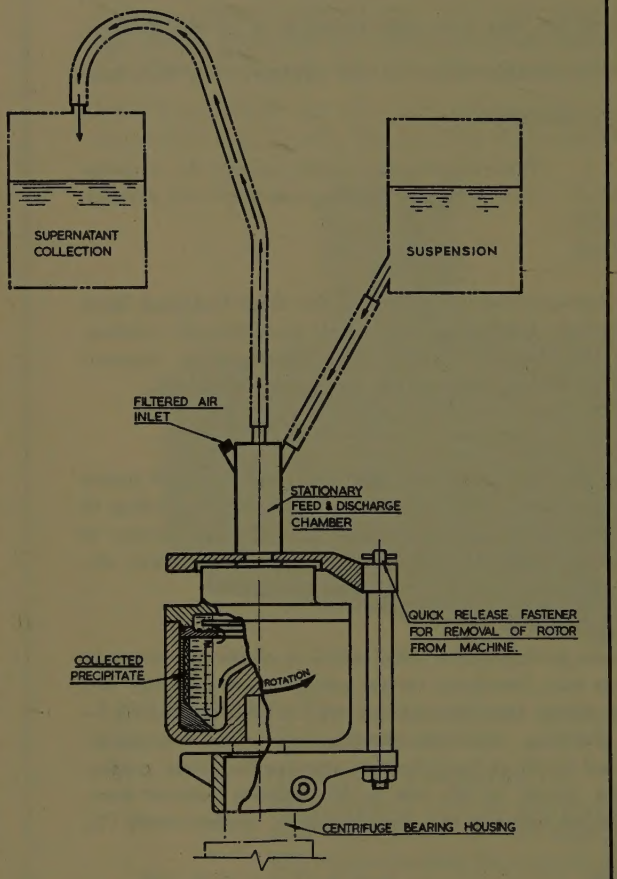

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DETERMINING THE pH OF A PHOSPHATE BUFFER SOLUTION FOR BLOOD MEASUREMENTS

By M. BAUMWOLL SPINNER and G. KOKHOLM PETERSEN

From The Department of Chemistry, Radiometer, Copenhagen, Denmark

(Received for publication May 30, 1960)

Measurement of pH in blood is being used re extensively in the determination of the vant quantities that characterize the acid- e status in blood.

uch determinations require equipment, n which a measurement can be repro- ed within ±0.005 pH with an absolute uracy better than ±0.01 pH.

The pH measurement is made by means of H-sensitive glass electrode and a calomel trode. It is a well-known fact that an ct measurement of pH by means of these trodes requires an adjustment of the elec- les against a buffer solution of known pH. n the course of time the pH has been ned in different ways and still to-day standardizing of the pH scale differs n one country to the other. Therefore it nly possible to compare two pH measure- ts if the method of measurement is the same and the buffer solutions used are prepared and checked in the same way.

The definition of the pH scale:

In 1909 S. P. L. Sørensen introduced a symbol of the "hydrogen ion concentration". It was defined as

$$p_H + = -\log c_H + \qquad (1)$$

where $c_H +$ is the hydrogen ion concentration.

Sørensen measured the $p_H +$ by measuring the emf of the cell:

Pt; H_2, test solution | salt bridge | 0.1 N calomel electrode. (2)

Sørensen measured the potential of the 0.1 N calomel electrode by measuring the emf of the cell (2) containing solutions of known hydrogen ion concentrations. The solutions were mixtures of hydrochloric acid

and sodium chloride. The concentration of hydrogen ion was assumed to be given by

$$c_H + = \alpha \cdot c \qquad (3)$$

where c is the normality of hydrochloric acid, and α is the degree of dissociation of the acid computed from measurement of electrolytic conductance, Kohlrausch and Holborn, 1898.

Based on this, Sørensen measured the $[p_H+]$ of a number of buffer solutions which have been and still are used extensively.

It is now generally recognized that the pH of an aqueous solution must be defined as the negative logarithm to the hydrogen ion activity

$$pH = -\log a_H = -\log f_H \cdot c_H \qquad (4)$$

where f_H is the activity coefficient of the hydrogen ion, and c_H is the concentration of the hydrogen ion.

However, the activity of a single ion is a concept which has no physical definition, so the establishment of a convenient pH scale leads to a conventional pH scale. Nevertheless, values found on the basis of this scale will, even if they are not true hydrogen ion activities when inserted in equations incorporating a_H, give results which are in reasonable agreement with those obtained by pure thermodynamic methods.

The conventional scale of activity:

The pH defined as

$$pH_X = pH_S + \frac{(E_X - E_S) \cdot F}{2.3026 \cdot RT} \qquad (5)$$

where E_X is the emf of the cell:

$$\text{Pt; } H_2\text{, soln. X } \Big| \text{ Saturated KCl solu., } Hg_2Cl_2\text{; Hg} \qquad (6)$$

and E_S is the emf of the cell (6), when the solution X has been replaced by a buffer o pH that is equal to pH_S. F and R are c stants, and T is the absolute temperature

In practice the hydrogen electrode generally replaced by a glass electrode w the same sensitivity as that of the hydrog electrode. Generally a liquid junction pot tial $E_{j,X}$, or $E_{j,S}$, occurs between soluti X or buffer solution, and the saturated K solution. In case

1) the pH lies between 3 and 11
2) the solutions are diluted

$E_{j,X}$ may often cause an error which d not exceed 0.02 pH. Moreover, if pH_X a pH_S are almost equal, and if the solution and the buffer solution are of uniform co position, $E_{j,X}$ and $E_{j,S}$ will be equal a therefore not involve errors. pH_S is pH i standard buffer solution.

METHODS

Some standard methods for the measurem

In U.S.A. (National Bureau of Standa (NBS)) five buffer solutions (see Table I) used for standardizing the pH scale. The pH the solutions has been measured by means of a without liquid junction potential.

In practice the pH is determined according equation (5) and the buffer solution used in standard buffer, the pH of which is nearest to p Thus the pH scale is defined in a series of po in a manner similar to that in which the temp ture scale is determined by a series of points.

According to British Standard, 1950 and No Francaise, 1953 the pH scale is only defined in point by means of one buffer solution, a 0.05 potassium hydrogen phthalate solution.

The difference between the pH of these meth for standardizing the pH scale depends on the of the sample, its composition and on the chara of the liquid junction. In the case of a diluted s tion the pH of which is between 3 and 11, difference will generally not exceed 0.02 pH.

Table I. *pH_S of NBS standards at 20° C.*

0.05 M potassium tetroxalate	1.68 pH
potassium hydrogen tartrate (saturated at 25° C)	3.56 pH (at 25° C)
0.05 M potassium hydrogen phthalate	4.00 pH
0.025 M potassium dihydrogen phosphate \ 0.025 M disodium hydrogen phosphate /	6.88 pH
0.01 M borax	9.22 pH

phosphate buffer solution for
od measurements:

enerally the pH in blood is close to 7.38 at C, so it is desirable to use a buffer solution ng a pH which is near this value.
ften a buffer solution is used which consists of:
1.816 g potassium dihydrogen phosphate
9.501 g disodium hydrogen phosphate, 2 H_2O
dissolved in water to give one liter.
reviously the pH of this solution was taken to .36 at 38° C, Astrup, 1956. To bring the pH his buffer in agreement with the conventional e of activity we measured the pH of the buffer g the method stated by NBS.
ccording to this method the pH of the buffer easured by means of a cell without a liquid tion potential:

H_2 (760 mm Hg), soln. X + Cl^-, AgCl; Ag (7)

known amount of sodium chloride is added to solution X whose pH is to be measured. The of the cell is measured for several concentra- and sodium chloride. A value called pwH is puted from:

$$pwH = \frac{(E - E_0) \cdot F}{2.3026 \cdot RT} + \log m_{Cl^-} \qquad (8)$$

re E is the emf of the cell (7), E_0 is the dard potential of the silver-silver chloride elec-e, and m_{Cl^-} is the molality of the chloride ion he solution X. The pwH^o, the value of pwH, the concentration of chloride ion is zero, is rmined by extrapolation. The pH of the solu-X is computed from:

$$pH = pwH^o + \log f^o_{Cl^-} \qquad (9)$$

re $f^o_{Cl^-}$ is the activity coefficient of the chlo- ion computed from the equation of Debye-kel. For further details, see R. Bates, 1954.

Reagents:

Potassium dihydrogen phosphate A. R., Merck, Disodium hydrogen photphate, 2 H_2O A. R., Merck, Sodium chloride A. R., Merck.

Apparatus:

The measuring apparatus was a precision compensation instrument made to give readings within ±0.01 mV. The measuring equipment consists of a constant temperature bath containing 4 cells (see Fig. 1). Each cell incorporates 4 platinized gold electrodes and 4 silver chloride electrodes (see

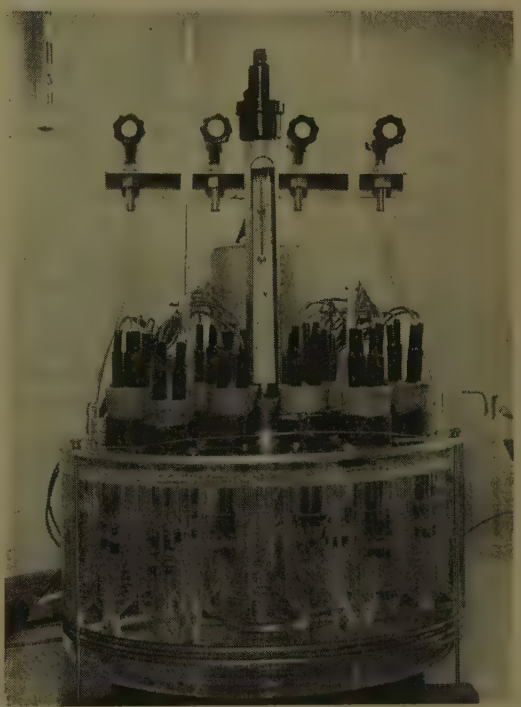
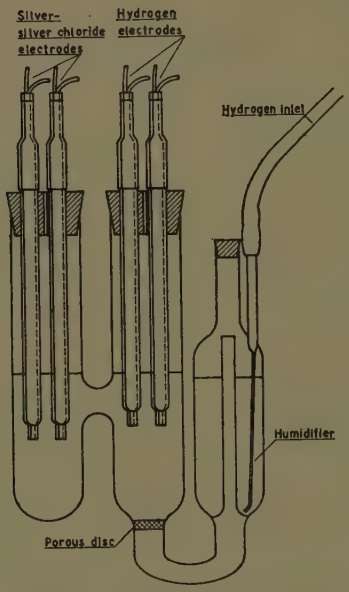


Fig. 1. Measuring-equipment.



.. Fig. 2. Measuring cell. ....

Fig. 2). The hydrogen gas used was purified by means of an oxygen purifier. The gas was saturated with water vapor at 38° C by being bubbled through a humidifier.

Procedure:

We measured the emf of the cell (7) for four concentrations of sodium chloride: 0.005, 0.010, 0.015 and 0.020 M. All solutions were made as molal solutions *i. e.* mol per 1000 grams water (the difference between the pH of the molal and the molar solution is negligible in this case). We used water with a conductivity of less than 1 μS.

The potential of each set of electrodes was watched for 6 hours. The potential was usually constant after about one hour.

RESULTS

The measurements are shown in Table II, which also shows the computation of the pwH values according to equation (8). The four pwH values indicate a straight line in a diagram where the concentration of chlori ion is the abscissa and pwH the ordinate (s Fig. 3). The straight line is computed b means of the method of least squares.

When the concentration of chloride ion zero, the value of pwH is found to be:

$$pwH^{o} = 7.5159 \pm 0.0005 \text{ at } 38.0^{\circ} \text{ C}$$

Because of errors due to the measuri equipment, which are not included in t standard deviation, the pwH° should be tak to be:

$$pwH^{o} = 7.516 \pm 0.002 \text{ at } 38.0^{\circ} \text{ C}$$

The pH of the buffer is computed from t equation (9).

Log $f^{o}_{Cl^-}$ is computed from the equati of Debye-Hückel:

$$-\log f^{o}_{Cl^-} = \frac{A \cdot z_i^2 \cdot \sqrt{\mu}}{1 + B \cdot a_i \cdot \sqrt{\mu}} = 0.1350 \quad (1$$

where a_i, the ion-size parameter, is taken be 4.4, and the ionic strength is found to 0.1735. A is 0.5202 and B is 0.3302

$$\underline{pH} = pwH^{\circ} + \log f^{\circ}_{Cl^-}$$
$$= \underline{7.381 \pm 0.002} \text{ at } \underline{38.0^{\circ} C}$$

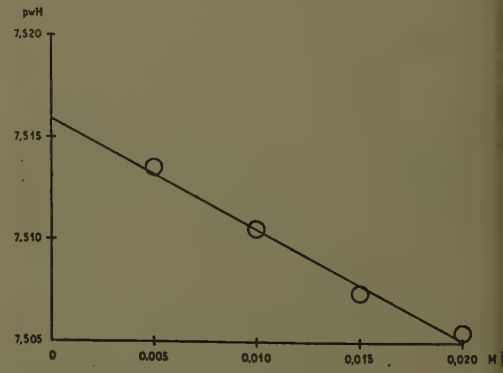


Fig. 3. Graph showing the relation between pw and the concentration of sodium chloride.

Table II.

E.: 213.52 mV			F: 96.4931	R: 8.31439 T: 311.16°		
Cl— conc. M	buffer batch No.	eltr. set No.	E 760 mm Hg mV	E (mean) 760 mm Hg mV	pwH	pwH (mean)
		1	819.51			
		2	819.51			
	1	3	819.51	819.52	7.5152	
		4	819.56			
		1	819.43			
		2	819.43			
	2	3	819.42	819.45	7.5140	
		4	819.51			
		1	819.38			
		2	819.41			
0.005	3	3	819.45	819.42	7.5135	7.5135 ± 0.0004
		4	819.42			
		1	819.49			
		2	819.50			
	4	3	819.48	819.49	7.5148	
		4	819.47			
		1	819.27			
		2	819.22			
	5	3	819.18	819.21	7.5102	
		4	819.15			
		5	800.82			
		6	800.79			
	1	7	—	800.80	7.5130	
		8	800.79			
		5	800.68			
		6	800.77			
	2	7	800.69	800.71	7.5115	
		8	800.71			
		5	800.65			
		6	800.43			
0.010	3	7	800.60	800.55	7.5090	7.5105 ± 0.0040
		8	800.53			
		5	800.64			
		6	800.64			
	4	7	800.62	800.65	7.5104	
		8	800.70			
		5	800.50			
		6	800.62			
	5	7	800.50	800.54	7.5088	
		8	—			

Table II (cont.)

Cl— conc. M	buffer batch No.	eltr. set No.	E 760 mm Hg mV	E (mean) 760 mm Hg mV	pwH	pwH (mean)
		9	789.62			
		10	789.78			
	1	11	789.76	789.70	7.5093	
		12	789.66			
		9	789.69			
		10	789.69			
	2	11	789.49	789.61	7.5079	
		12	789.55			
		9	789.46			
		10	789.63			
0.015	3	11	789.56	789.55	7.5067	7.5074 ± 0.0003
		12	789.56			
		9	789.45			
		10	789.57			
	4	11	789.51	789.51	7.5061	
		12	789.49			
		9	789.54			
		10	789.61			
	5	11	789.61	789.57	7.5071	
		12	789.53			
		13	781.77			
		14	781.75			
	1	15	781.78	781.76	7.5055	
		16	781.75			
		13	781.76			
		14	781.67			
	2	14	—	781.72	7.5048	
		16	781.74			
		13	781.79			
		14	781.78			
0.020	3	15	781.77	781.80	7.5062	7.5055 ± 0.0004
		16	781.85			
		13	781.86			
		14	781.88			
	4	15	781.89	781.89	7.5076	
		16	781.92			
		13	781.60			
		14	781.59			
	5	15	781.62	781.62	7.5032	
		16	781.67			

SUMMARY

ıe pH of a phosphate buffer solution for
l determinations is measured by a meth-
imilar to the method for standardizing
H scale used by the National Bureau of
dards, U.S.A.

ıe pH of a buffer solution containing
5 g potassium dihydrogen phosphate
l g disodium hydrogen phosphate, 2 H_2O
1000 grams of water was found to be

1.381 ±0.002 at 38.0° C.

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SERUM PROTEINS AND PROTEIN-BOUND CARBOHYDRATES IN TUBERCULOSIS AND AMYLOIDOSI

By E. LAUSING[1] and C. RYDE

From the Department of Clinical Chemistry, University Hospital, Uppsala, and the Sandträsk Sanatorium, Sandträsk, Sweden

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Amyloidosis is a relatively frequent complication to long-standing pulmonary tuberculosis and to other chronic diseases with suppuration and tissue destruction. Generally, the amyloidosis is considered to be a disorder of protein metabolism, its pathogenesis is discussed in detail *inter alia* by Johansson (1954) and Teilum (1954). Changes in the serum protein distribution in man suffering from pulmonary tuberculosis and from secondary amyloidosis have been the subject of several investigations (Seibert, Seibert, Atno & Campbell 1947, Lezgus 1951, Perä-salo & Louhimo 1955, Puls & Albaum 1952). The most constant changes in the electrophoretic pattern seem to be a decreased albumin and increased α_2- and γ-globulin, the alterations in other globulin fractions being variable. Likewise, alterations in the content of serum protein-bound polysaccharides and their electrophoretic distribution have been a subject of interest (Seibert, Nelson & Seibert 1943, Seibert, Seibert, Atno & Campbell 1947, Seibert, Pfaff & Seibert 1947, Puls & Fiedler 1956, La rence, Weimer & Salkin 1959).

In the present study serum from a numb of cases of advanced pulmonary tuberculo and of secondary amyloidosis was analys with respect to total protein, protein-bou hexose and the electrophoretic distributi of protein and protein-bound polysacchari in order to investigate whether any speci protein changes occurred in these diseas Special attention was given to the questi whether some more characteristic chan appear when advanced and long-standi pulmonary tuberculosis is complicated secondary amyloidosis.

MATERIAL

Our material, collected at the Sandträsk Sa torium, includes 69 cases of advanced pulmon tuberculosis and 10 cases of secondary amyloido The cases of tuberculosis have been divided two groups: moderately advanced and far advan tuberculosis. The number of cases in the first gr was 36 and in the second 33. This classification been made with the purpose of studying whet the changes in serum protein and glycoprotein c tent and electrophoretic pattern become more centuated when the clinical picture of the diseas

[1] Present address: Central County Hospital, Karlstad, Sweden.

fulminant and severe. For this classification -ray findings have been decisive. Considera- as been paid to the extent of the pulmonary tions, to their character — exudative or pro- e — and to the occurrence of caverns and hiectasia. But also other clinical signs of the of the process such as body temperature, ., weight gain or loss have been taken into leration. As in all clinical evaluations this fication may be somewhat overlapping. The ion of the disease after diagnosis varyied en 6 months and 30 years. There was no ation between the severity of the clinical e and the duration of the disease. All the ts in these groups have been treated with culostatic drugs. In the group of moderately ced tuberculosis one patient had albuminuria. g the cases with far advanced tuberculosis patients had moderate albuminuria, one of had total serum protein less than 6 g/100 ml. the amyloidosis cases the diagnosis has been med by autopsy. These cases were divided two groups: without albuminuria and with inuria. In the first group there were 4 pa- with blood samples analysed 8 times. The n duration of the tuberculosis in this group rom 1 to 6 years, and the pulmonary changes far advanced. Autopsy revealed amyloid de- ation of varying degree in liver and spleen. of the cases had moderate degeneration in the ys, in the remaining two these organs were from amyloid deposits. In the group with inuria there were 6 cases, investigated 8 times. duration of tuberculosis was from 4 to 20 and in all cases the pulmonary lesions were dvanced. Albuminuria in these cases had been ntly present for at least one year prior to the blood samples, the amount of albumin ne varying from 0.04 to 0.7 g per 100 ml. All ese cases had at autopsy a severe amyloid eration of liver, spleen, and kidneys.

normal material a group of 30 healthy adults th sexes was used. All serum samples were at —20° C until analysed.

METHODS

tal serum protein was determined by a ied biuret method according to Heyrowsky & rova (1951). The protein-bound hexose was determined by Björnesjö's (1955) modification of the anthrone method.

Electrophoretic separation of serum proteins and protein-bound polysaccharides was carried out *ad modum* Aronson & Grönwall (1957) on Schleicher and Schüll 2043 B paper using their TEB buffer of pH 8.9. The potential gradient was 6.5 v/cm and the running time 16 hours. The strips were stained for protein with amido black 10 B (Grassman, Hanning & Knedel, 1951) and for polysaccharides with Schiff's reagent according to Aronsson, Grönwall & Lausing (1959). The strips were evaluated with Spinco Analytrol using a green filter with a transmission maximum at 540 mμ and cam B 3.

RESULTS AND DISCUSSION

The total serum protein presented in Table I was within normal limits in the two groups with tuberculosis and in secondary amyloidosis without albuminuria. In amyloidosis with renal impairment there was, however, a clear decline in the serum protein content. As for the cases with tuberculosis, the results agree with those of other investigators. The values for serum protein in amyloidosis published by other authors are more variable. Wald (1955) reported normal values for 21 out of 28 cases of secondary amyloidosis with albuminuria, Cohen (1943) found total serum protein decreased in 53 per cent of his amyloidosis cases.

The electrophoretic distribution of serum protein is shown in Fig. 1, the results being given as relative distribution and as the absolute amount of protein in each fraction, calculated as mean values for each group. The $\pm$ 2.5 δ limits for normals are marked by horizontal lines.

There was no change in the prealbumin fraction. The albumin fraction showed a

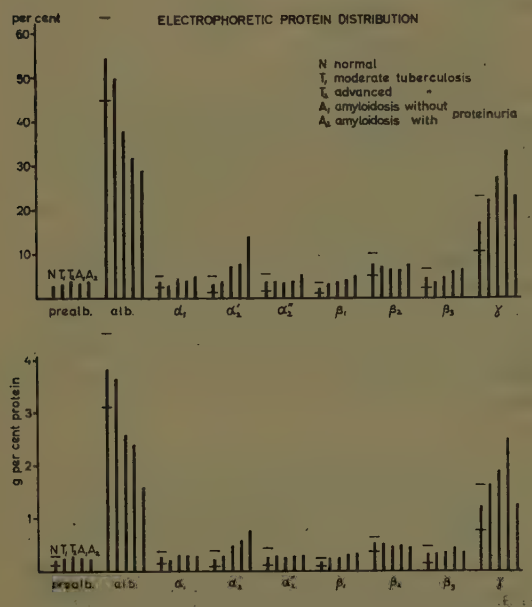

Fig. 1. Electrophoretic distribution of serum protein in normals, in tuberculosis and in secondary amyloidosis.

gradual decrease. It was still within normal limits in moderately advanced tuberculosis, the decrease being clear in far advanced tuberculosis and most marked in amyloidosis with albuminuria The decrease in the last groups is certainly aggravated by renal loss of protein. There was no abrupt change between far advanced tuberculosis and amyloidosis without albuminuria.

As for globulin fractions, the detectable changes were found in the fast moving α_2 fraction, in β_1- and in γ-globulin, the alterations in other globulin fractions not exceeding the $\pm$ 2.5 δ limits for normal mean values. The increase in the fast α_2-component became apparent in far advanced tuberculosis and showed a further increase in amyloidosis groups. Using TEB buffer serum haptoglobin migrates in this fraction and is elevat in acute and chronic diseases (Nyma 1959). In the present study no determinati of haptoglobin was made and it is therefo not possible to discuss to what extent hapt globin or other α_2-globulin subfractions c tribute to this increase. In contrast to t increase of the fast α_2-compound, the slow α_2-fraction showed no change.

Of the β-globulin subfractions the β_1-gl bulin showed an increase which was mo prominent in amyloidosis. There was a slig tendency of decrease in β_2- and increase β_3-globulin. The γ-globulin fraction show an increase in far advanced tuberculosis a in amyloidosis without albuminuria. T changes in γ-globulin seem to parallel t severity of the disease and the tendency increased total serum protein in amyloido without proteinuria, as shown in Table seems to be due to increased α_2- and γ-g bulins.

In amyloidosis with renal impairment t γ-globulin is decreased compared with t group without albuminuria. The changes electrophoretic patterns of protein in am loidosis with albuminuria are somewh different from those met in nephrosis other etiology (Sunderman & Sunderm 1957) where the characteristic changes considered to be: an increase in α-globu and decrease of all other fractions. T normal γ-globulin fraction in amyloid phrosis might be a result of the very lo content of that fraction before the develo ment of renal damage.

The content of serum protein-bou hexose is shown in Table I. It was increas in tuberculosis and amyloidosis, the incre being slight in moderately advanced tub

Table I. *The content of serum protein, hexose and hexose to protein ratio in normals, in tuberculosis and in secondary amyloidosis.*

	Protein g/100 ml	Hexose mg/100 ml	Hexose Protein x 100
Normal	7.0 (6.2—8.1)	128 (98—143)	1.8 (1.4—2.1)
Moderately advanced tuberculosis	7.3 (6.3—8.4)	148 (104—193)	2.0 (1.6—2.6)
Far advanced tuberculosis	6.8 (5.8—8.4)	201 (140—255)	3.0 (1.9—4.3)
Amyloidosis without proteinuria	7.6 (6.4—9.8)	224 (185—268)	3.0 (2.5—3.4)
Amyloidosis with proteinuria	5.4 (3.3—6.7)	206 (168—237)	3.8 (2.9—5.1)

s and most marked in amyloidosis ut proteinuria. There was a consider- overlapping of hexose values in tuber- s groups, but the lowest values in amy- is were above the +2.5 δ limit for als. The content of hexose in protein correspondingly from 1.8 per cent in als to 3.8 per cent in amyloidosis with nuria. It was further noticed that the of protein in that group apparently ed those protein fractions poor in e, as the remaining protein is more rich xose compared with serum in amyloi- without proteinuria.

e results of the electrophoretic separa- of protein-bound polysaccharides are n in Fig. 2, the results being presented ative distribution and as content of pro- ound hexose in different protein frac- Regarding the polysaccharide content umin, the reports found in the literature According to Cohn, Hughes & Weari) crystallized albumin is practically from carbohydrate. Müller-Eberhard (1956), using the anthrone reaction, has shown that the carbohydrate content in electrophoretically separated albumin is very

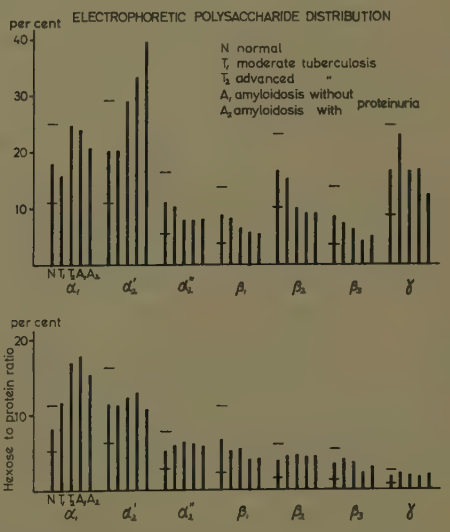

Fig. 2. Electrophoretic distribution of protein-bound polysaccharides and hexose to protein ratio in normals, in tuberculosis and in secondary amyloidosis.

low. Using the periodic acid-Schiff reagent for the staining of electrophoretically separated protein-bound polysaccharides, a distinct coloration of the albumin fraction is obtained when the separation is carried out on filter paper. However, the observation that there is no or a weak Schiff positive reaction at the albumin zone when using cellulose acetate "Oxoid" strips, and other not yet published investigations (Aronsson, 1959) from this laboratory indicate that a part of this staining of the albumin zone on filter paper is not due to polysaccharides but is unspecific. For this reason the albumin zone has been excluded and the evaluation of the polysaccharide strips was started with the α_1-globulin fraction.

The relative distribution of polysaccharides showed an increase in α_1- and in the fast-moving α_2-fraction and a decrease in other globulin fractions. As these changes in relative distribution are influenced by total protein-bound hexose content in serum and by changes in protein content in fractions, it is more convenient to consider the changes in the per cent of hexose content in different protein fractions. There was only one protein fraction that had become more rich in polysaccharide, namely the α_1-globulin, the increase exceeding the $\pm$ 2.5 δ limits for normal material in tuberculosis and in amyloidosis. The greatest rise took place between the two tuberculosis groups and there was no marked change when amyloidosis had developed. This finding indicates that a qualitative change has occurred in the composition of α_1-globulin and suggests an inhomogencity of this fraction. The hexose content in other protein fractions did not show any significant changes, a tendency to decrease was noticed in the β_1- and β_3-globulin fracti
The relative increase of polysaccharide
the fast-moving α_2-globulin parallels alm
entirely that of the protein. It might
pointed out that, of the two α_2-globulin fr
tions with nearly equal protein content
normal serum, the fast-moving fraction
twice as rich in protein-bound hexose as
slower one. Contrary to reports on car
hydrate content in γ-globulin in experime
tuberculosis in animals (Sher, Dubin, Ta
mure & Popper, 1957) and in tubercul
in man (Puls & Albaum, 1956), the chan
in our material were not significant,
hexose content in the elevated γ-globulin
tuberculosis and amyloidosis being somew
lower but of the same magnitude as in nor
cases.

SUMMARY

69 cases of pulmonary tuberculosis
10 cases of amyloidosis secondary to tu
culosis were analysed for total protein,
tein-bound hexose and electrophoretic dis
bution of serum proteins and polysacchari
In cases of tuberculosis and of amyloid
the electrophoretic serum pattern showe
decrease in albumin, an increase in the f
moving α_2-globulin and in β_1- and γ-
bulin fractions. These alterations were
sentially the same but more marked in a
loidosis. The content of protein-bound he
in serum was increased, the rise being
prominent in amyloidosis with renal imp
ment. The hexose content of the diffe
protein components showed an increas
α_1-globulin, the changes in other fract
being slight.

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THE ASSAY OF PROPERDIN

AN EVALUATION OF THREE METHODS

By Ø. ØVSTHUS and J. BØE

From the Medical department B, Haukeland sykehus, University of Bergen, Bergen, Norway

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Pillemer's discovery of the properdin system (Pillemer, Blum, Lepow, Todd & Wardlaw 1954) started a veritable avalanche of investigations concerning this system and its rôle in the unspecific resistance. It did naturally appear very promising that a factor, perhaps an important factor, in this unspecific resistance had been found and that it could be measured quantitatively.

Later investigations and the literature regarding the properdin system, which is by now very extensive, have clearly shown that there is no such simple relationship between the properdin content of blood and unspecific resistance as indicated by Pillemer's earliest investigations (Pillemer *et al.* 1954, Pillemer, Schoenberg, Blum & Wurz, 1955, Pillemer, Blum, Lepow, Wurz & Todd, 1956).

Gradually, it has also become clear that properdin determinations in blood (serum) present many difficulties of a technical nature, so many that several investigators have abandoned the method. The results obtained have partly been difficult to reproduce and partly shown little agreement with the clinical findings.

A considerable number of methods have appeared for the estimation of properdin blood (Leon 1956, McNall 1957, Isliker Linder 1958, Fritsche, Fischer, Schwick Schultze 1958, Willers, Pondman & Win ler 1959). Most of these are modifications Pillemer's original method (Pillemer *et* 1954, Pillemer *et al.* 1956) based on t principle that properdin combines wi zymosan, (an insoluble carbohydrate) give an insoluble complex (PZ) that is ab to inactivate the third component of compl ment (C'3) at 37°, but not at 17°. In a pr perdin-free serum (RP), no inactivation C'3 by zymosan takes place, but inactivati will occur when both zymosan and properd are added at 37°.

During some years work on properdin d terminations in connection with infectio diseases, we have had the opportunity work with and compare three different met ods for the analysis of properdin, and o experience as to the advantages and disa vantages of these methods is presented he

METHODS

The three methods for properdin assays to compared here are all zymosan methods, nam Pillemer's method (Pillemer *et al.* 1954, Pille *et al.* 1956), a modification by Willers *et al.* (195 and one by Leon (1956).

llemer's method

test tubes containing 0.6 mg zymosan each, dded decreasing amounts of serum starting 0.1 ml in the first tube. Thereafter 0.1 ml RP nt is added to each tube, and the volumes are up to 0.3 ml with barbital buffer pH 7.4. ubes are incubated 1 hour at 37°, 1 ml sen- l sheep cells (1.25 per cent) is added to each ie incubation is continued for 30 min., where- the tubes are centrifuged for 5 min. at r.p.m.

lemer defined 1 unit of properdin as that ity which, in the presence of an optimal nt of zymosan, completely inactivates 120 ± its of C'3 in 1 ml of RP during 1 hour at 37° mer *et al.* 1956).

cording to this, the end point in the titration d as the mean value of the test serum amount last tube showing no hemolysis and the first showing a trace of hemolysis. The number of rdin units in the sample is determined by ng this mean value into 0.1 (amount of RP to each tube).

illers' method

e test serum is incubated with the same nts of zymosan and RP reagent as for the us method, 75 min. at 17°. The tubes are then fuged, the supernatant poured off, and the itate (PZ complex) is washed with barbital- . Fresh RP is added, and the tubes are in- d 1 hour at 37°, whereafter sensitized sheep re added as for 1.

using this procedure a PZ complex is formed , and any inhibitors present in the test serum might influence the inactivation of C'3 by removed before this inactivation takes place . This technique also provides a known and nt C'3 content in all the test tubes before the ation of C'3 in the RP reagent occurs, in- lent of the test serum dilution as this is ed beforehand.

vided any inhibitors influence the inactivation by the PZ complex only, and not the forma- f the PZ complex itself, Willers' method will what is termed "total" properdin in serum, ie properdin titer obtained with Pillemer's technique will represent "available" properdin, this titer being dependent on the amount of inhibitors present in the test serum.

3. *Leon's method*

The test serum is incubated with zymosan only, 75 min. at 17°, centrifugation and washing are carried out as for 2. RP reagent is then added, the tubes are incubated 1 hour at 37°, and sheep cells are added as before.

Unknown cofactors required for the reaction of properdin *in vitro* may also be necessary for its *in vivo* reactions. In omitting the addition of RP reagent (containing the necessary cofactors) during the incubation at 17°, one obtains a properdin titer dependent on the test serum's concentration of cofactors, "available" properdin. According to this theory the two previous methods discussed here will give the "total" properdin content in serum[1].

Reagents

1) *Veronal buffer, pH 7.4* (Kabat, 1948)

85.0 g NaCl
5.75 » 5.5 — diethylbarbituric acid
3.75 » sodium 5.5 — diethylbarbiturate
5.0 ml M $MgCl_2$
1.5 » M $CaCl_2$

The ingredients are dissolved in about 1500 ml of hot distilled water, and after cooling the volume is made up to 2 liters with distilled water.

Immediately before use one part of this solution is diluted with four parts of distilled water.

2) *Zymosan suspension*

1 g of zymosan[2] is suspended in 1000 ml. 0.15 M NaCl and boiled in a waterbath for one hour. The suspension is centrifuged and the residue is resuspended in 1000 ml diluted veronal buffer. This

[1] Since the completion of this work we have been aware of Laurells modification of the properdin assay (Acta path. et microbiol Scandinav. *45*, 186, 1959). Apart from the incubation temperature this method seems similar to that of Leon described here.

[2] Zymosan, Type A, was obtained from the Fleischmann Laboratories, c/o Standard Brands Inc., Special Products Division, 625 Madison Avenue, New York 22, N.Y., U.S.A. Batch 7B — 152 and 7B — 340 were used for this work.

suspension is activated before use by boiling in a waterbath for ten minutes, whereafter 0.6 ml is added to each test tube to be used for the assay. The tubes are centrifuged for 5 minutes, and the supernatant is poured off.

3) *R 3 reagent*

This reagent is not required for the properdin assay itself but is necessary for titration of the C'3 content in the RP reagent used for the titration of properdin. It is prepared according to Pillemer (1953) by removing C'3 and properdin from fresh serum in treating this with zymosan at 37° C.

4) *RP reagent*

The RP is prepared from fresh serum by removal of properdin with zymosan at 17° C. At this temperature zymosan will combine with properdin without inactivating C'3 and the PZ-complex may be removed by centrifugation. 1 mg zymosan per ml serum is suspended in the serum and it is placed a waterbath at 17° C for one hour stirring eve ten minutes. After centrifugation fresh zymosan added to the supernatant, and the treatment repeated.

The resulting RP reagent will in most cases free of properdin, but will not always satisfy t other requirements for a good RP for properd titrations. The RP must have a complement (titer and C'3 titer of not less than 75 per cent the parent serum, and the C'3 content should 120 ± 30 C'3 units per ml.

When the RP is treated with zymosan at 3 for one hour, the C'3 titer should not decrea more than 25 per cent. Further, the RP must be anticomplementary against fresh serum, and must contain all the cofactors necessary for t formation of PZ and the inactivation of C'3 by P also adequate amounts of C'1, C'2 and C'4 hemolysis of sensitized sheep cells.

Table I. *Properdin levels of normal sera assayed with three different methods.*

Serum	Properdin units per ml serum		
	Pillemer's Method	Willers' Method	Leon's Method
1	<1	3	3
2	<1	1,5	2
3	<1	3	3
4	<1	3	3
5	<1	3	<1
6	<1	<1	–
7	<1	<1	–
8	<1	8	–
9	<1	7	–
10	1.5	3.5	1.5
11	3	6	7
12	3	7	8
13	3	6	6
14	3	7	7
15	3	3	3
16	5	7	5
17	5	7	5
18	5	7	7
19	6	6	6
20	7	8	–
	(Mean properdin titer 2.5 units)	(Mean properdin titer 5 units)	(Mean properdin titer 4.5 units)

The same RP reagent was used for the three methods

Table II. *Properdin levels of some pathological sera.*

rum	Diagnosis	Properdin units pr. ml serum		
		Pillemer's Method	Willer's Method	Leon's Method
1	Pneumonia	<1	<1	<1
2	»	<1	<1	<1
3	»	<1	3	2
4	»	3	7	7
5	»	4	4	5
6	Hepatitis acuta	<1	6	2
7	Nephritis	<1	5	<1
8	Leukemia	<1	3	2
9	Lupus eryth. diss.	<1	7	2
10	Meningitis (Haem. infl.)	<1	3	<1
11	Septicemia (Staf. aur.)	7.5	7.5	7.5
12	Abscessus peritons.	7	7	7
13	Angina faucium	2.5	7.5	7
		(Mean properdin titer 2.4 units)	(Mean properdin titer 5 units)	(Mean properdin titer 3.2 units)

The same RP reagent was used for the three methods

titrations of complement and its components rried out as described by Kabat (1948).

nsitized sheep cells

ep blood is kept in an equal volume of r's solution (Kabat, 1948). Before use, a por- centrifuged and washed twice with diluted veronal buffer. The packed cells are then suspended in diluted veronal buffer to give a 2.5 per cent suspension, whereafter this is mixed with an equal volume of diluted buffer containing 4 units of hemolysin (Kabat, 1948).

Reagents 1) and 2) should be kept in the refrigerator while R3 and RP must be stored in frozen condition.

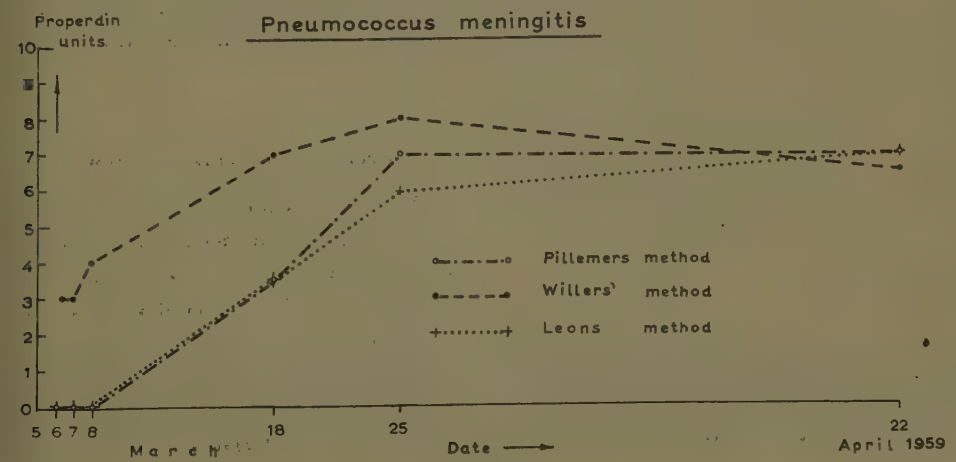

Fig. 1.

Table III. *A comparison of the methods of Pillemer and Willers using different RP reagents.*

Serum	Properdin units per ml serum							
	Pillemer's Method			Willers' Method				
	RP I	RP I	RP II	RP I	RP I	RP I	RP I	RP II
1	1	1		3	3	3		3
2	1	1		3	3	3		3
3	1.5	1.5		3	3	3		
4	3	3		4	5	5		5
5	3	3		<4	<4	3		3
6	3	3		6	5	5	5	5
7	1.5		1.5			4	3	3

All sera were ultracentrifuged at 33 000 g for 2 hours

RESULTS

a) *Properdin levels (height of titer)*

By applying these three methods to normal sera at various properdin levels, utilizing the same RP reagent for the three methods, one finds as a rule that Pillemer's method gives lower results than the other two (Table I) while Willers' method generally gives the highest values.

If one examines pathological sera in the same way, the findings are very similar (Table II). More often than not, Willers' method gives the highest properdin values, and if one follows the change in the properdin content as it may be seen during the course of a serious infectious disease (Fig.1), this method shows the increase in properdin at an earlier stage than the other two.

b) *Repeatability*

Our experience with repeatability of the assays is that one has to accept at least a one-tube variation (Tables III and IV).

The constant titer one finds in a patient again and again may also be used as a mea ure for the repeatability of the metho (Fig. 2).

It should be noted that the real difficul in performing the tests is to prepare suitab reagents (properdin-free sera, RP), and th the number of control tests in our mater by far outnumbers the properdin determin tions in sera from pathological conditions. however, suitable RP reagents are at han the repeatability of these three methods fairly high (Tables III and IV) where t tests are performed with different RP agents.

c) *Effect of ultracentrifugation*

As it is possible that high molecular su stances present in serum may interfere w the properdin and C'3 assays (Landy Pillemer 1956), ultracentrifugation has be applied to several of our samples, as this remove the substances in question (Table

The table shows that in our experime no change occurred in the titers after

Table IV. *Repeatability of Pillemer's zymosan assay for properdin using different RP reagents.*

Serum	Properdin units per. ml serum Pillemer's Method						
	RP III	RP III	RP III	RP III	RP IV	RP VI	RP VI
1	<1	<1			<1		
2	<1	<1					
3	<1	<1			<1		
4	<1	<1			<1		
5	<1	<1			<1		
6	<1	<1					
7	1.5	<1	<1	<1	<1		
	RP II	RP II	RP V	RP V			
8	3	4			4		
9	5	3			5		
10	3	3	3				
11	4	3				5	
12		5	4.5			3	4
13			3.5	5		5	4
14			3			3	3
15				5		3	4
16						3	3

All sera were ultracentrifuged at 33 000 g for 2 hours

sera had been centrifuged at 33,000 g for 2 hours. We did, however, find that in some cases where agreement in titers could not be obtained, this presented no difficulties after the samples had been centrifuged at high speed.

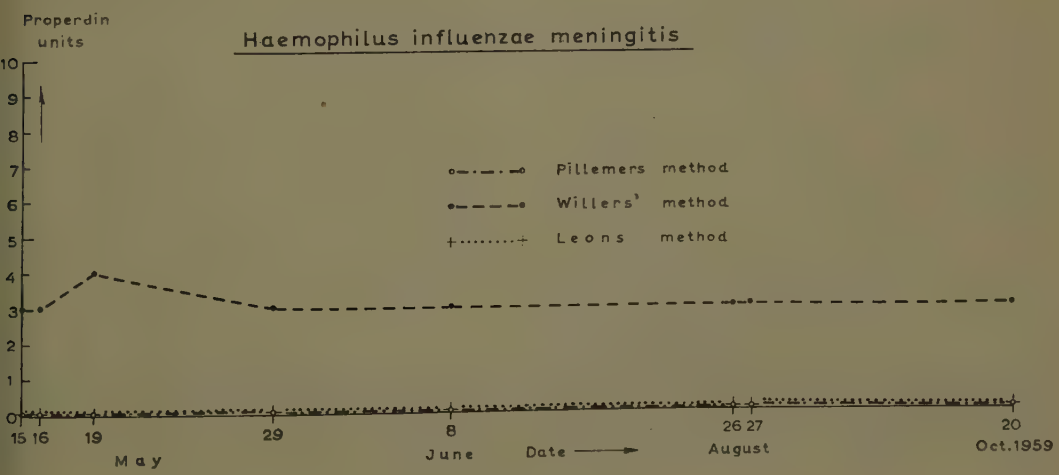

Fig. 2.

Table V. *Properdin levels of human sera before and after ultracentrifugation at 33,000 g for 2 hours.*

Serum	Diagnosis	Pillemer's Method		Willers' Method	
		Before	After	Before	After
1	Normal	<1	<1	3	3
2	»	1	1	4	4
3	Meningitis (Meningoc.)	<1	<1	<1	<1
4	» (Haem. infl.)	<1	<1	3	3
5	Pneumonia	<1	<1	<1	<1
6	»	<1	<1	3	3
7	»	<1	<1	3	3
8	Endocarditis subac. bact.	<1	<1	<1	<1
9	Morbilli	<1	<1	<1	<1
10	Polyarthritis chron.		7	8	8
11	Colitis ulcerosa	<1	<1	3	3
12	Lupus eryth. diss.	<1	<1	8	7

DISCUSSION

When a comparison is to be made of the three methods of assay, one finds that they all have both advantages and disadvantages. Common for the three is the difficulty in obtaining sufficient and satisfactory reagents. The preparation and testing of serum reagents for the properdin assay require much more work than the actual titration of samples. Also a considerable number of control tests must be carried out. When suitable reagents are available, the assay itself is fairly simple to perform.

As to the time required for each method, Pillemer's is the quickest, while both Willers' and Leon's methods demand at little more time.

The repeatability is about the same for the three assay methods, and is sufficiently high to enable one to determine the properdin titer in serum, and also to follow any variations in the properdin content of serum. Willers' method seems to have one advantage in preference to the others as it generally gives higher titers, and any increase in the pro perdin content of serum may be registere at an earlier stage. It appears, in using thi technique, easier to follow the fluctuation of the properdin levels in blood during pa thological conditions, particularly as regard results which with the Pillemer techniqu will be considered as lack of properdin or a subnormal values, while Willers' techniqu in these cases often will give a definite tite Titers obtained with Willers' method als give the impression of being easier to rea as it gives more clear cut changes than th other two. A drawback with this method i however, the use of twice as much RP r agent as is necessary for Pillemer's an Leon's methods, a rather serious drawbac when one considers the difficulty in obtai ing this reagent.

Which titer should be considered as th "correct" one in this connection, and whic reflects the clinical condition better than th others is as yet unknown. It is possible th the factor of importance is not the properd

ntent as such, but the "available" pro-
rdin or even unknown inhibitors (Willers
al. 1959). If this is so, Willers' method
ill not reflect the clinical condition as it
iminates the influence of any inhibitors.

Pillemer's method, on the other hand,
ould express the "available" properdin
er, while the presence of inhibitors will be
tected only in comparing the results
tained with both these methods.

It is also possible that unknown cofactors
e of importance (Willers *et al.* 1959, Sou-
r & Ménaché 1958), in which case Leon's
ethod will give the "available" properdin,
d a comparison of Leon's and either Pille-
er's or Willers' method would be necessary
r the detection of any cofactors.

The clinical importance of properdin de-
rminations has so far not come up to the
pectations raised at the publication of
illemer's earliest work and generalizations
ncerning properdin and natural resistance,
t our results do suggest that during the
urse of certain infections, the properdin
vels may reflect the clinical condition. If
e properdin determination should become
importance in the clinic, it might be poss-
le to centralize the preparation of the
cessary reagents in the same way as has
en done for the coagulation factors of the
ood (Owren 1959). If one is under the
cessity of making ones own reagents, the
nclusion must be that the methods avail-
le at present for determination of proper-
n are unsuitable for routine use in a
boratory.

SUMMARY

Three methods of properdin assay, the
illemer method, the modification by Leon
d that of Willers *et al.* are evaluated.

Preference is given to the method of Willers. It is the easiest to read, gives the highest titers and in pathological conditions, where the titer may rise from subnormal to normal values, the rise comes first and is most consistent with this method. It has the serious drawback that it requires more of the reagents than the other two methods.

The real obstacle in all three methods is the difficulty in preparing suitable reagents. This, in addition to the numerous control tests required, makes the assay of properdin too elaborate for routine laboratories.

ACKNOWLEDGEMENT

This work was supported by the Norwegian Research Council for Science and the Humanities.

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POSTMORTEM ABSORPTION OF CARBON MONOXIDE IN RATS

By J. E. ASVALL

Institute of Aviation Medicine, Royal Narwegian Air Force Oslo, Norway

(Received for publication April 24, 1959)

Carbon monoxide intoxication has for ıny years been an important problem in iation medicine. Nuttal (1958) found 34 ses with pathological amounts of carbon ɔnoxide among all intoxications reported ıong the personnel in US Air Force in the ear period 1955—57, and the recent re-rt of such poisioning of the pilot of a ɔrwegian civil airliner demonstrated that s danger hos not yet been eliminated, even modern aircrafts.

The diagnosis of carbon monoxide in-ɪications has long been based on the ɛrmination of the amount of carboxy-moglobin (COHb) in the blood, but later dies have also brought forward a method tissue analysis for use in those cases where od is not available. It has been shown Vilks, 1956) that this method gives satis-tory results in human material when com-ed with simultaneous blood examinations. S Air Force Institute of Pathology has for ne time performed routine analyses of ody extracts from tissue (by the same thod as for blood) to find any sign of CO-oxication of persons involved in aircraft idents, and Siliphant & Stembridge (1958) report that of 134 cases, pathological values were found in 14, all of which had been in conjunction with fire, usually following the crash of the plane. They concluded that the significance of this investigation was that an increased content of COHb found post mortem indicated a premortem absorption of CO, either by in flight intoxication or by death *after* the onset of fire usually following airplane crashes. This evaluation of the significance of post mortem demonstration of increased COHb content seems to be a generally accepted point of view; we have not found any workers who doubt its value.

The Institute of Aviation Medicine, Royal Norwegian Air Force, has carried out analyses for COHb in the blood of all pilots involved in accidents or near-accidents since February 1957. Up to December 1958 a total of 65 cases had been investigated, but only one bloodsample revealed pathological amounts of COHb. This sample had been taken at autopsy from the aorta of a pilot dead in the burned-out wreck of his jet-fighter, and the subsequent analyses revealed a COHb content in the blood of 22 %. According to the generally accepted theory

this should demonstrate a premortem CO absorption. However, there was no significant story that made pre-crash CO intoxication likely, and the severe injuries found in the head and body as a result of the impact made even a short-time post-impact survival improbable. This problem then led us to the presumption of a postmortem absorption of CO produced by the burning wreck, as a possible explanation for the pathological findings. To investigate this theory the following experiments were done.

METHODS

To obtain an indication of the quantity of CO absorbed by the animals during the experiments, the amount of COHb in blood was determined. The analytical method applied is based on the original work of Hüfner (1900), giving the amount of COHb compared with the total amount of hemoglobin, expressed as a percentage. Modifications of Hüfners method have been made by different workers; with some minor changes the technique described by Harboe (1957) has been used in these experiments.

In outline, the method is as follows: About 50 microliters of blood are expelled into 10 ml 0.04 per cent NH_3 and stirred with a glass rod, then centrifuged for 4—5 minutes at 3000 rpm. The centrifugate is placed in two standard cuvettes, and the optical density of each is read at 500 and 541 mμ at a predetermined temperature. The ratio D_{541}/D_{560} is calculated, and with this ratio the COHb per cent is read from a calibration curve. Originally the density at 576 mμ was also read, and the ratio D_{576}/D_{560} used for calculating the COHb content. However, the work of Harboe (1957) showed that this ratio was less stable in samples stored for several days and also showed somewhat poorer correlation with the gasometric method of Scholander & Roughton (1943) than did the ratio $D_{541}/_{560}$. For these reasons it was not considered necessary to take readings at 576 mμ.

Each of the COHb values presented in this paper is the mean of 2 parallel determinations (as indica ed by the 2 standard cuvettes used for each blo sample). To determine the reliability of the meth in our hands, 17 samples from the same blood w analyzed with 2 parallel determinations each. T arithmetic mean of the COHb content was fou to be 24 per cent with a standard deviation of 0

When any COHb content was found during t experiments, the content of methemoglobin in t blood was estimated by the method of Evelyn Malloy (1938), and a correction for this was ma

EXPERIMENTS

Rats weighing 250—300 grams were used f these experiments; sexdifference was not tak into consideration. Before the experiments the r were anesthezised with ether, and a bloodsamp was taken from a tail vein in order to determi the presence (if any) of COHb before the expos to the CO-containing gas. The animals were th divided into 5 experimental groups:

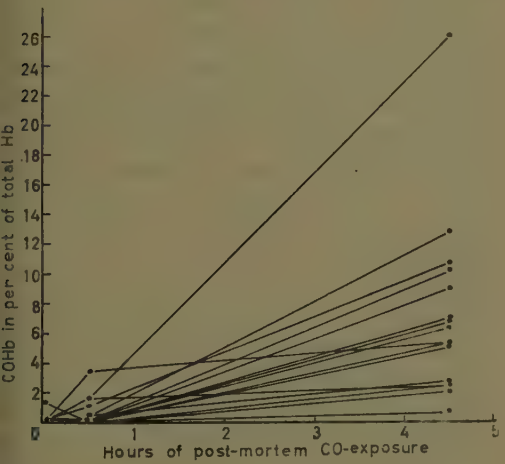
Experiment 1: The rat was killed by an ov dosage of ether and left for 5 minutes to all postmorted convulsions to cease. The tongue th was pulled forward, and the mouth was kept op by forcing a small glass ring between the jaws order to secure open airways to the lungs. The was placed in a small chamber (volume abo 8000 cc) into which led a tube introducing 8 lit per minute of coal gas containing 12.3 vol. cent CO. After ½ hour in this atmosphere the was removed, the thorax opened and a card puncture in the right atrium performed. Great c was taken not to contaminate the sample with CO-containing air outside the heart. To prev this the samples were taken with Pasteur pipet with very fine, capillary ends. These were sto outside the room where the rats were placed, a each pipette was thrown away after having b used once. Care was taken not to squeeze pipette sucker more than absolutely necessary allow the suitable amount of blood to enter at introduction of its end into the atrium. With end of the pipette within the heart the sucker allowed to expand slowly and a close watch kept on the blood rising in the pipette to disco

y sign of simultaneously introduction of air, in hich case the sample was immediately discarded. fter the sampling the thorax wound was loosely osed, and the rat was exposed to CO in the amber for another 4 hours, after which a second rdiac puncture was made.

16 rats were treated this way. Fig. 1 shows the sult of the COHb determinations. All the animals sorbed some CO during the last 4 hours of posure, giving a mean final value of 6.4 per cent OHb as shown by Fig. 2 (exp. 1).

Experiment 2: To find out whether the CO sorbed had entered by diffusion through the skin, rats were killed and 2 "surgeon's fingertips" lled over the head of each rat. These fingerstalls tally prevented any CO gas from entering through e mouth or nose, making absorption through the in the only possible way. The rats were then posed for a full 4½ hour period in the gas amber before the first and only cardiac puncture as performed. The final absorption was negligeble, with a mean COHb as shown in Fig. 2 exp. 2).

Experiment 3: In the second part of experiment o. 1, the rats were exposed with a loosely closed



g. 1. 16 rats killed by overdosage of ether. Carxyhemoglobin values in blood taken premortem ero time) and after ½ and 4½ hours of postmortem CO exposure.

wound in the thorax during the last 4 hours of CO exposure. To test importance of this, experiment No. 2 was repeated, but with the alteration that

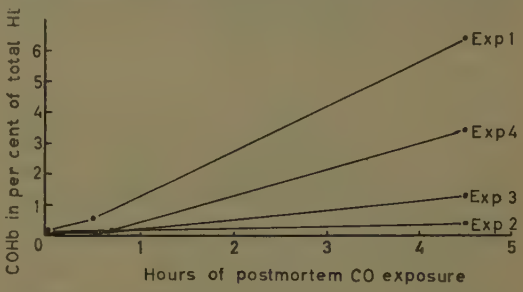


Fig. 2. Carboxyhemoglobin values in blood from rats taken premortem (zero time) and after ½ and 4½ hours of postmortem CO exposure. The curves represent the arithmetic mean of experimental groups 1—4 respectively.

before the exposure period a thorax wound was produced and then closed in the same manner as when the cardiac punctures in the previous experiments were performed. The group of 20 rats showed a mean final absorption equalling 1.3 per cent COHb, as indicated by Fig. 2 (exp. 3).

Experiment 4: To make conditions more similar to those usually found in aircraft accidents, 27 rats were killed by a hard blow on the head, making them bleed through the nostrils and down the pharynx. Five minutes later they were put in the gas chamber in random position, some with their neck bent or twisted. Cardiac puncture was performed after ½ and 4½ hours of gas exposure. Fig. 3 show that 2 of the rats had an exceptionally high absorption during the first ½ hour of exposure, while their COHb formation during the last 4 hours was comparable to the rest of the group. This is most probably caused by postmortem convulsions with subsequent irregular inspirations; a common feature in this type of death in rats. The mean final absorption for the other 25 rats was 3.4 per cent COHb, as shown by Fig. 2 (exp. 4).

Experiment 5: To check whether death itself might produce substances which interfere with the COHb determination, 5 rats were used as controls. These were killed in the same way as those in

exp. 4 and placed in random position in ordinary fresh air. After ½ hour and 4½ hour cardiac punctures were performed. None of these blood samples showed any absorption in the measured wavelengths resembling COHb.

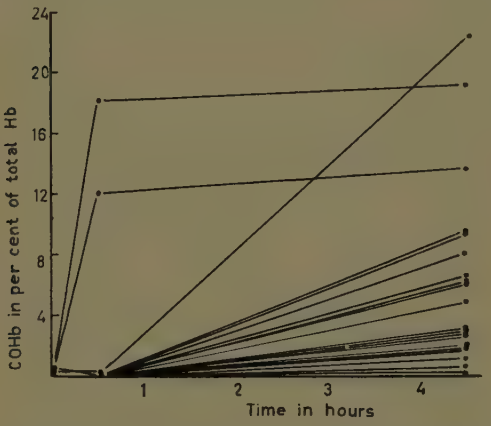


Fig. 3. 27 rats killed by *trauma capitis*. Carboxyhemoglobin values in blood taken premortem (zero time) and after ½ and 4½ hours of postmortem CO exposure.

DISCUSSION

Fig. 2 seems to indicate a difference in degree of absorption for experiments 1—4. To prove whether this is really so, a statistical test of significance has to be carried out. However, this requires first and foremost that the groups to be compared belong to the same type of distribution. In order to determine this, Fig. 4 and Fig. 5 have been made to show the frequency of the different values (*i. e.* the histograms) for COHb found in exp. 1 and exp. 4 respectively. The curve in Fig. 4 differs in shape from the one in Fig. 5; therefore one is not allowed to state that the 2 groups represented by these curves belong to the same type of distribution — though this is not excluded; an increase in the number of observations may yield another result. The conclusion must be that these data do not allow further statistical evaluation.

However, by studying Fig. 2 it seems likely that the absorption is caused mainly by pulmonal diffusion subsequent to passage of CO down the respiratory tract. The difference between experiments 1 and 4 is most probably the result of partial obstruction of the airways and alveoli by blood from the epipharynx of those animals killed by *trauma capitis*.

Compared with the concentration of the CO in the coal gas and the duration of the CO-exposure (12 per cent CO for 4½ hours) the quantity of carbon monoxide absorbed seems to be very low. With one exception the COHb values for the 83 rats in groups 1—4 are all under 12 per cent at the end of 4½ hours exposure, with a mean for the highest group of 6.5 per cent. It is difficult to estimate what this means in terms of actual CO absorption. According to Krogh (1915), Cushny was correct in considering the alveolar wall as being physiologically a watery membrane, thereby making the absorption of CO a matter of pure diffusion.

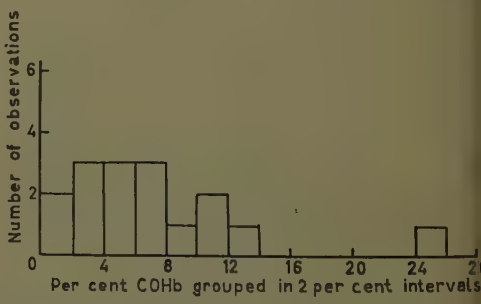
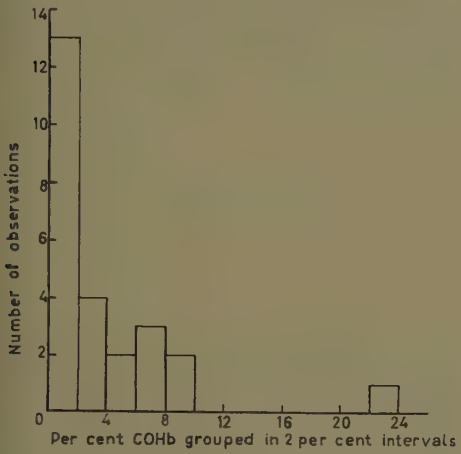


Fig. 4. 16 rats killed by overdosage of ether. Carboxyhemoglobin values after 4½ hours of postmortem CO exposure, grouped in per cent intervals.

he degree of this would be proportional to ie gas tension throughout the tissue, and ius factors controlling the diffusion would ot be likely to change much immediately fter death. If this is so, a COHb of 6.4 per



ig. 5. 25 rats killed by *trauma capitis*. Carboxy-emoglobin values after 4½ hours of postmortem CO exposure, grouped in 2 per cent intervals.

ent would correspond to a CO tension in ie pulmonal tissue of less than 0.1 ‰, ccording to Douglas & Haldane (1912).

As a conclusion the results show, with the eservation necessary in all animal experi-ients, that post mortem absorption of CO oes not usually take place to any significant egree. However, CO-formation in gas from urning fuel can be rather high, up to 30 per ent (Drinker, 1938); though the pro-ability of maintaining such a high concen-ation for a long period in an actual fire is from a burning aircraft) is not great. aking into consideration the spread of the dividual values found, postmortem absorp-on should nevertheless be kept in mind in ses where pathologic amounts of COHb found at autopsy are difficult to explain, and there is likelihood that the corpse has been exposed to carbon monoxide.

SUMMARY

The post mortem absorption of carbon monoxide has been studied in rats. After 4½ hours exposure to coal gas containing 12 per cent CO one experimental group showed a mean increase of carboxyhemoglobin in heart blood of 6.4 per cent; the other groups gave lower values. The individual spread in results was considerable, but only 1 rat out of a total of 83 had a level above 13 per cent COHb. It is concluded that the post mortem absorption of CO is not likely to occur to any significant degree, at least when the CO avaiable in the atmosphere does not exceed 12 per cent.

ACKNOWLEDGEMENTS

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ON THE SERUM ACTIVITY OF LACTIC ACID DEHYDROGENASE AND PHOSPHOHEXOSE ISOMERASE IN PERNICIOUS AND HEMOLYTIC ANEMIAS

By C. GRÖNVALL

From the Department of Clinical Chemistry, General Hospital, Malmö, Sweden

(Received for publication June 17, 1960)

Lactic acid dehydrogenase (LDH) occurs varying concentrations in the different lular systems of the body. The main source LDH normally occurring in serum is not t known. Wieland & Pfleiderer (1957) ve shown that LDH from different organs not represented by identical proteins.

Increased serum LDH[1] activity has been served in a number of diseases, including ocardial infarction (Wróblewski 1957, ick & Blumenthal 1956), different types leukemia (Wróblewski 1957, Zimmerman Weinstein 1956, Hsick & Blumenthal 56), muscular trauma (Wróblewski 1957), er diseases (Wróblewski 1957, Zimmer- n & Weinstein 1956, Hsick & Blumenthal 56), renal diseases (West & Zimmerman 58), different types of anemia (Wróblew- 1957, Hess & Gehm 1955, Lührs & Ne- ein 1956, Zimmerman & Weinstein 1956, nmerman, West & Heller 1958, Gordin Enari 1959, Cintrón-Rivera, Acosta-Matienzo & Diaz-Rivera 1959, Heller, West & Zimmerman 1959, Levitan, Wasserman & Wróblewski 1959), general carcinomatosis (Wróblewski 1957, Lührs & Negelein 1956, Zimmerman & Weinstein 1956, West & Zimmerman 1958, Hsick & Blumenthal 1956). Exceptionally high values have sometimes been noted in vitamin B_{12} deficiency, but it appears that no attempts have been made to find an explanation of this phenomenon.

In 16 cases of pernicious anemia (p.a.) Hess and Gehm (1955) found serum values 5—21 times normal. Lührs and Negelein (1956) describe two patients with p.a., in whom the LDH was 8 times the normal values. In 12 cases of megaloblastic anemia, including 7 with p.a., Zimmerman *et al.* (1958) found LDH to be only 2—3 times the upper normal limit. Gordon and Enari (1959), on the other hand, observed markedly raised values in p.a. and pernicious tapeworm anemia. During vitamin B_{12} therapy the values rapidly returned to normal.

The red blood cells contain LDH in a

[1] Hereafter the abbreviation LDH will be used denote the recorded activity of lactic acid de- rogenase.

concentration about 220 times that in the serum, but in hemolytic anemia, both acquired and congenital, only slightly raised or normal values have been found in the serum (Zimmerman & Weinstein 1956, Zimmerman *et al.* 1958).

Phospho hexose isomerase (PHI), which catalyses the conversion of fructose-6-phosphate to glucose-6-phosphate and *vice versa*, also occurs in the serum with abnormally high activity[1] in a number of pathologic conditions, including acute hepatitis (Bruns & Hinsberg 1954), leukemia (Blanchaer, Green, Maclean & Hollenberg 1958), cancer (Bodansky 1954, Bruns & Jacob 1954), diabetes mellitus (Bruns & Jacob 1954) and megaloblastic anemia (Heller, West & Zimmerman 1959).

In the present investigation the LDH in the serum from patients with p.a. was compared with that in hemolytic anemia. In some of the patients the serum PHI was also determined.

In 4 patients with p.a. the activities of these enzymes in the plasma were studied during remission after B_{12} therapy.

MATERIAL

The material consisted of sera from 27 cases of p.a. and 16 cases of hemolytic anemia.

The diagnosis p.a. was based on the demonstration of megaloblastic bone marrow in all cases except 2, where no typical megaloblasts were found, but where the concentration of vitamin B_{12} in the serum was subnormal (see Table II). The correctness of the diagnosis was confirmed further by the presence of histamine refractory achylia, and in some cases by the result of the Schilling test and by remission during the course of B_{12} therapy.

In 10 cases with p.a. the LDH was determi in the serum as well as in the red blood cells.

The hemolytic anemias consisted of 6 cases congenital hemolytic anemia, 3 cases of hemol anemia caused by phenacetin and 7 cases of acqui hemolytic anemia of unknown origin.

The normal material for determination of LI and PHI in the serum consisted of sera from and 54 blood donors respectively. Whole blood fr 29 apparently healthy persons was used as nor material for determination of LDH in the blood cells.

METHODS

LDH in the serum and the red blood cells. The LDH was determined according to Wrobl ski and La Due (1955). The LDH in the red bl cells was calculated on the basis of the hemato and LDH values of serum and whole blood. In last mentioned determinations the red blood c were hemolysed by addition of water.

PHI in the serum. — The PHI was determi according to Bodanski (1954). The activity is pressed in units differing from Bodanski units. determination according to Bodanski the amount fruktose-6-phosphate (F-6-P) formed is de mined by Roe's fructose determination method before every determination calibration is d with standard solutions of F-6-P. To simplify method, fructose was used as standard in the sent investigation. One unit is to be understood the F-6-P formed, which gives the same extinc on determination of fructose according to Roe, 1 microgram of fructose.

RESULTS

It is clear from Table I that in pernici anemia as well as in hemolytic anemia LDH and PHI in the serum are increas The relative deviation from the normal v ues was greatest for LDH in both conditio Fig. 1 gives the serum LDH in relation the hemoglobin content of the blood in

[1] Hereafter the abbreviation PHI will be used to denote the activity of phosphohexose isomerase.

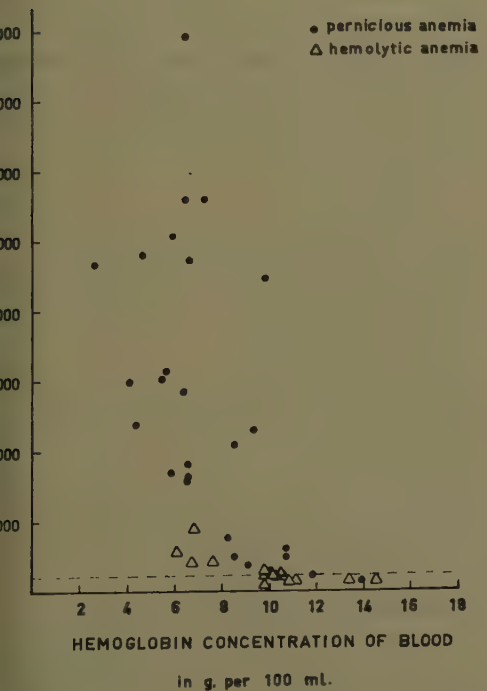
Table I.

	Number	Serum LDH in U./ml./min.		Serum PHI in U.	
		Mean	Range	Mean	Range
rmals*	53	266	110—422	—	—
nicious anemia	27	5360	300—15900	—	—
molytic anemia	15	575	140—1770	—	—
rmals*	54	—	—	7.0	2.1—11.9
nicious anemia	14	—	—	30.3	11.3—45.2
molytic anemia	9	—	—	8.9	5.6—16.7

*For the normals the range is given as the mean ± 2 standard deviations.

lividual cases. The activity was higher en the concentration of hemoglobin was v than when it was high, and at the same ncentration of hemoglobin it was higher in cases of p.a. than in cases of hemolytic anemia. The same tendency was found for PHI (Fig. 2).

Values for the 2 cases of p.a. without



. 1. The serum LDH in relation to the hemobin content of the blood. The line marks the upper normal limit of serum LDH.

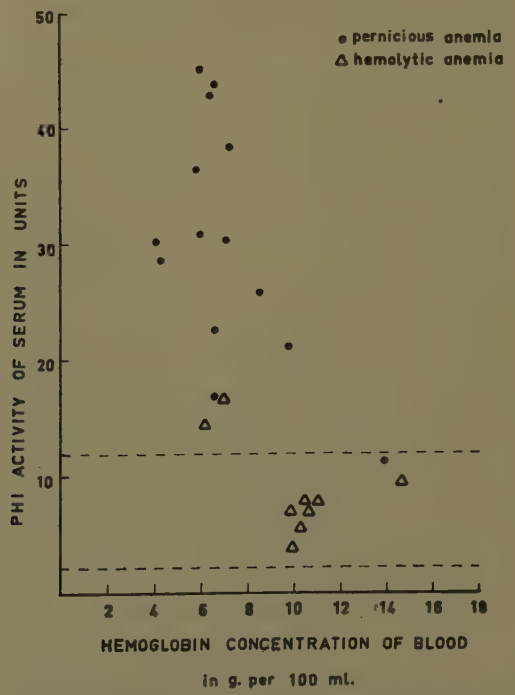


Fig. 2. The serum PHI in relation to the hemoglobin content of the blood. The lines mark the normal limits of serum PHI.

Table II.

B_{12} in serum Picograms/ml.	LDH in serum U./ml./min.	PHI in serum U.
25	1000	—
25	300	11.3

The values of the 2 cases of p.a. without megaloblasts in the sternal puncture.

megaloblasts in the sternal puncture are given in Table II.

In 4 cases of p.a. the serum LDH and PHI were followed during treatment with vitamin B_{12}. A temporary rise (see Figs. 3 and 4) was sometimes noted, after which the activity for both enzymes decreased rapidly.

The LDH was determined in 10 cases of p.a. also in the red blood cells. The me value was 67 700 and the lowest a highest values were 31 000 and 100 0 U./ml./min. respectively. For the norm material the mean was 76 000 and the norm range (mean ± 2 standard deviation 60 000—92 000 U./ml./min. The differen between the means was not significant, the range of variation was clearly wider p.a. than in the normals.

DISCUSSION

The serum concentration of an enzy depends on the ratio between the inflow a the elimination of the enzyme, but when t concentration of the enzyme is determin indirectly by measurement of the activity, t value obtained is influenced by the presen of any activators or inhibitors. Substances

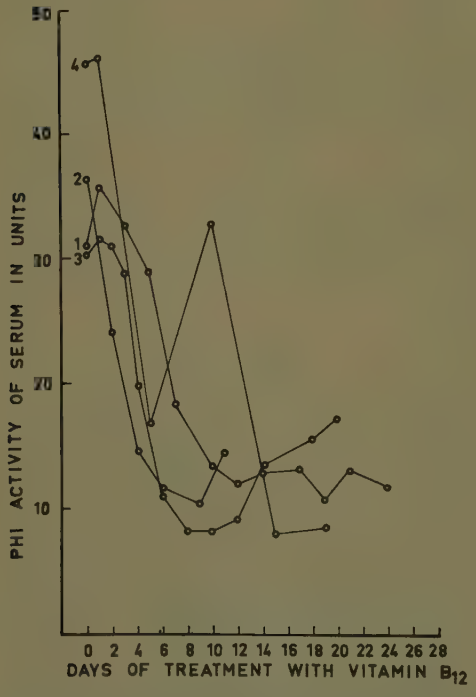


Fig. 3. LDH in serum during treatment of pernicious anemia with vitamin B_{12}.

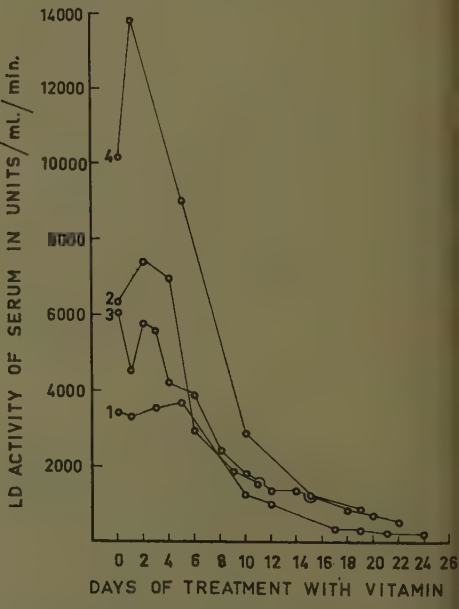


Fig. 4. PHI in serum during treatment of p nicious anemia with vitamin B_{12}.

rum from healthy persons and from pa-
nts, which inhibit or activate LDH and
HI have not been studied in further detail.
is generally believed that the activity of
ese enzymes in the serum largely reflects
e concentrations.

It is clear from the results that the increase
LDH was excessive in advanced cases of
., in contrast to what is found in advanced
molytic anemia, where the serum values
e admittedly very pathologic, but of the
me order as in myeloid anemia and ad-
nced liver disease, for example. The PHI
lues showed the same trend but the
anges were less pronounced.

In anemia, serum values exceeding 3000
/ml./min. for LDH could be regarded as
thognomic of vitamin B_{12} deficiency, but
ey are of less diagnostic value because the
rly cases with only a slight lowering of the
mber of red blood cells in the circulation
ow moderate or no LDH increase in the
rum.

The LDH content in circulating red blood
lls in patients with p. a. is of the same order
in normals. The markedly increased in-
w of LDH into the blood stream in p.a.
ay be due to increased rate of destruction
red blood cells. In p.a. a slightly shortened
rvival time of the red cells can be demon-
rated with Cr-tagging of the red blood cells,
t the plasma iron turnover, the stercobilin
cretion and the endogenous CO-formation
ow that the total hemoglobin turnover must
much greater than what is obtained on
lculation according to Cr-tagging. A con-
lerable so-called bone marrow hemolysis is
obable. In the normal metabolism of aged
d cells as in most hemolytic anemias, on
e other hand, the main destruction of the
blood cells occurs extravascularly (Jandle, Jones & Castle 1957; Noyes, Bothwell & Finch 1959).

If the rate of elimination of the LDH molecule from the blood stream, the LDH in the serum and the LDH concentration in the red blood cells are known, it is possible, according to the following formula, to calculate how large the daily intravascular hemolysis must be for maintenance of the LDH activity found in the serum if it is assumed that all serum LDH emanates from erythrocytes and that the distribution volume of LDH is equal to double the serum volume.

Formula

per cent hemolysis of circulating blood cell mass

$$\frac{2 \cdot \frac{0.693}{T/2} \cdot (1\text{-Hkr}) \cdot LDH_{serum} \cdot 100}{Hkr \cdot LDH_{erythrocytes}}$$

where $T/2$ = half time of serum LDH in days

Hkr = hematocrit

A maximum value for the half time of serum LDH was calculated graphically for 4 cases of p.a. during treatment with vitamin B_{12}. Only the LDH values recorded 4 days or more after commencement of therapy were included. The pathologic increase of the LDH inflow in, or the pathological decrease of the LDH elimination of the serum then appeared to have stopped. The fall in the LDH values in relation to the time agree then with the elimination according to a simple exponential course. The value found for the half time, 3 days, gives approximately the highest possible true value.

Table III.

LDH, U./ml./min.		Per cent daily hemolysis of circulating blood cells for maintenance LDH in serum	Calculated survival time in days
in serum	in erythrocytes		
440	31000	0.20	500
650	42000	0.17	588
1150	75000	4.09	24
790	72000	1.26	79
1250	70000	2.08	48
1000	97000	1.52	66
11500	100000	31.16	3.2
5000	81000	10.66	9.3
490	46000	0.87	115
510	59000	1.13	88

The steady state of LDH in the plasma of normals corresponds to a daily hemolysis of the circulating red blood cell mass of 0.36 per cent, which corresponds to an average erythrocyte survival time of 277 days, if the serum LDH emanates mainly from destroyed red blood cells — an improbable assumption. The survival time is normally about 120 days, which corresponds to a daily hemolysis of 0.83 per cent. From the value obtained it can be concluded that the bulk of LDH in the blood cells that undergoes normal, extravascular hemolysis is not returned in active form to plasma.

Corresponding calculations were made in 10 cases of p.a. where LDH was determined in the serum and red blood cells and where the hematocrit value was known. Results are given in Table III. In the 8 cases of p.a. with the highest serum LDH according to Fig. 1, similar calculations were made. The hematocrit and LDH in the erythrocytes were not determined in these cases. Therefore, the normal value for LDH in the erythrocytes, 76 000 U./ml./min., was taken. The hemat crit values were calculated from the numb of erythrocytes per mm^3 on the assumpti that the mean volume of the megalocytes w 1.3 times that of normocytes. Calculatio showed that in these 8 cases of p.a., mainte ance of the serum LDH would require a dai *intravascular* hemolysis of 16—18 per cent the circulating erythrocytes on the assum tion that LDH emanated solely from the r blood cells. Values of the same order are al given in Table III. They correspond to mean survival time of the erythrocytes 6.2—0.15 days and are thus unreasonab This shows that the increased activity LDH in the serum in p.a. emanates not o from the hemolysed circulating red blo cells. The molecular weight of the LDH probably more than 100 000, so a more less general cell leakage is improbable. T results suggest that the very high LD values in serum in advanced B_{12} deficien reflect severely disturbed cellular functi probably with shortened cell survival in t

ne marrow (*e. g.* megaloblasts) or in other sues.

The pathways by which the LDH and HI are eliminated are not known, nor is elimination mechanism. Evidence from mal experiments (West & Zimmerman 58) suggests that the LDH is normally not creted in the urine. It cannot be excluded *priori* from the present investigation that directly or indirectly affects the elimination rate and that the high level of LDH in serum in a steady state in B_{12} deficiency y be explained by a slower elimination.

SUMMARY

Serum activity of lactic acid dehydrogenase DH) and phosphohexose isomerase HI) were studied in cases of hemolytic emia and pernicious anemia. The latter owed very high relative increase in activity both enzymes compared with normal. lues of LDH exceeding 3000 U./ml./min. anemia argue strongly for pernicious anea. The half time of LDH in serum was termined approximately in cases of perious anemia during treatment with vitan B_{12}. On the basis of the results the ssible cause of increased serum LDH are cussed.

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FIBRINOLYTIC ACTIVITY PRODUCED IN GUINEA-PIG SERUM BY SOME HUMAN BODY FLUIDS AND BY HYALURONIC ACID

By E. S. OLESEN

From the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark

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The fibrinolytic properties of human body ids have been the subject of several invest- tions (for references see Albrechtsen 59). The observation that fibrinolytic ivity can be elicited in guinea-pig serum peptone (Ungar & Mist 1949, Olesen 57 a, 1959 a) as well as by acid poly- charides (Olesen 1959 b) and other onic polyelectrolytes (Olesen 1959 c) ggests a possible mechanism through ich body fluids containing similar sub- nces might produce fibrinolytic activity the organism. The present investigation nonstrates that some human body fluids produce effects on the fibrinolytic system guinea-pig serum which are comparable the effects reported for peptone and onic compounds. Some preliminary obser- ions on the effect of human hydrocele d, hygroma fluid, amniotic fluid and va have previously been mentioned (Ole- 1957 b).

MATERIALS AND METHODS

Pooled samples of guinea-pig serum were kindly supplied by Dr. P. Krag of the Danish State Serum Institute. Human body fluids were obtained from hospital patients. Serum and the fluids were kept frozen at —20° until use and between experiments.

Samples were prepared by isoelectric precipitation as follows: Serum, body fluid, or mixtures of these two (0.9 per cent NaCl to correct volume) were diluted with distilled water to 20 times the total volume and adjusted with 0.2—0.5 per cent acetic acid to pH 5.4 (unless otherwise stated). After centrifugation, the precipitates were dissolved in saline barbital buffer (0.05 M diethylbarbital, 0.1 M NaCl, pH 7.75). These solutions were examined for fibrinolytic activity on bovine fibrin plates (0.1 per cent fibrinogen) according to Astrup & Müllertz (1952), and for protease activity on fibrin plates heated at 85° for 40 min. according to Lassen (1952). Bovine thrombin was kindly supplied by the Leo Pharmaceutical Co., Copenhagen. Fibrinolytic activity was expressed as the diameter product (mm^2) of the lysed zones after 18 hours incubation at 37° (average of 3 determinations).

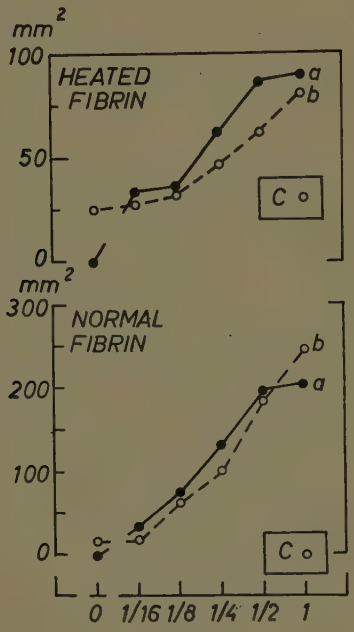


Fig. 1. Fibrinolytic activity after isoelectric precipitation at pH 5.4 of (a) guinea-pig serum containing various amounts of hydrocele fluid, or (b) hydrocele fluid containing various amounts of guinea-pig serum; (c) hydrocele fluid directly applied.

Abscissa: ml of hydrocele fluid per ml of serum, or *vice versa* (logarithmic scale).

Ordinate: Fibrinolytic activity as diameter product (mm^2) of lysed zone.

RESULTS

Hydrocele fluid, added in increasing concentrations to guinea-pig serum before isoelectric precipitation at pH 5.4, produced increasing fibrinolytic activity of the precipitates (Fig. 1, curve a). A precipitate separated from the hydrocele fluid showed only minimal activity, but an increase followed when guinea-pig serum was added before the precipitation (Fig. 1, curve b). Hydrocele fluid, directly applied to the fibrin plates, produced almost the same slight lyt effect (Fig. 1, c) as did isoelectrically pr cipitated hydrocele fluid. The activiti recorded on normal and heated fibrin pr sent a fair degree of correspondance. T lysis of heated fibrin indicates that an acti protease, presumably plasmin, is present the test samples.

When precipitations were performed various pH-values (see Fig. 2), hydroc fluid in this experiment showed no demo strable fibrinolytic activity (the concentr tion of the solutions was half that tested the above experiment). Guinea-pig seru yielded some activity at pH 6.2—6.6; but

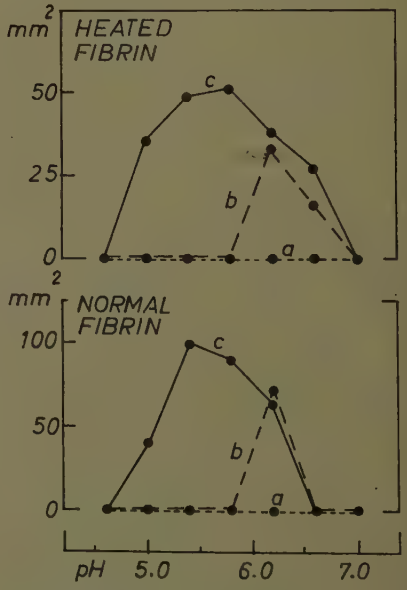


Fig. 2. Fibrinolytic activity after isoelectric pre pitation at different pH-values of (a) hydroc fluid + saline, (b) serum + saline, and (c) hyd cele fluid + serum (equal volumes).

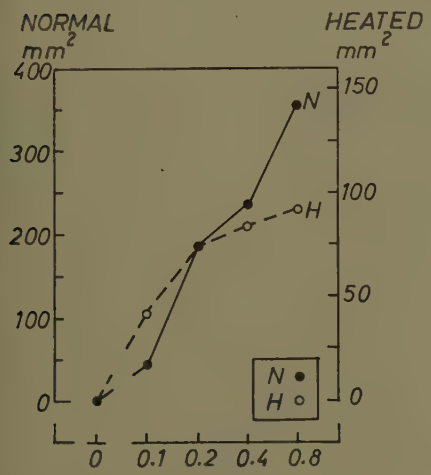
Abscissa: pH.
Ordinate: Fibrinolytic activity.

xture of serum and hydrocele fluid pro-ced higher activities, with maximum at a re acid reaction, pH 5.4—5.8.

Fluid from a prepatellar *hygroma,* and id from a knee joint *hydarthron* produced similar effect (Fig. 3 and Fig. 4): When se fluids were added to guinea-pig serum fore isoelectric precipitation at pH 5.4, ey caused a greater increase in the fibrino-ic activity of the precipitates than could expected from the activities obtained by ecipitation of the respective fluids sepa-ely.

The sample of hydarthon fluid in itself (isoelectrically precipitated) produced considerable lysis of the fibrin plates; the activity was relatively high on heated fibrin, indicating that it was largely due to a protease (Fig. 4, frame). When the amount of hydarthon fluid added to 1 ml of guinea-pig serum was increased beyond 0.2 ml, it produced a further increase in activity on normal fibrin without a corresponding increase in protease activity on heated fibrin (Fig. 4). This additional gain in activity was probably caused by activation of the plasminogen present in normal bovine fibrin.

Amniotic fluid (cleared by centrifugation)



. 3. Fibrinolytic activity after isoelectric pre-itation at pH 5.4 of guinea-pig serum with the lition of hygroma fluid. Inserted in frame: ivity of hygroma fluid alone, isoelectrically pre-cipitated.

scissa: ml of hygroma fluid per ml of serum (logarithmic scale).

dinate: Fibrinolytic activity. Different scales for ivity on normal and heated fibrin, (N) and (H), respectively.

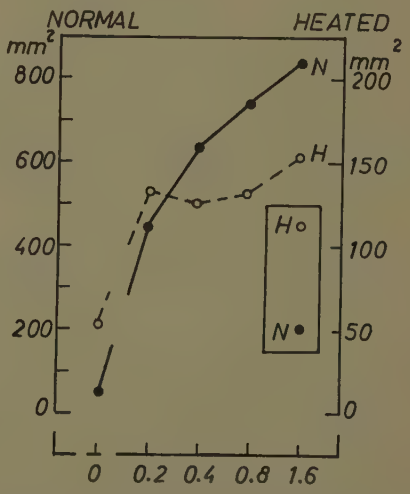


Fig. 4. Fibrinolytic activity after isoelectric precipitation at pH 5.4 of guinea-pig serum with the addition of hydarthron fluid. In frame: activity of hydarthron fluid alone, isoelectrically precipitated.

Abscissa: ml of hydarthron fluid per ml of serum (logarithmic scale).

Ordinate: Fibrinolytic activity. Different scales for activity on normal and heated fibrin, (N), and (H). respectively.

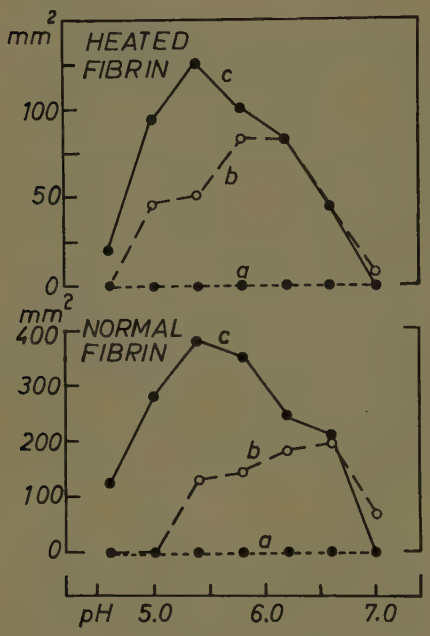


Fig. 5. Fibrinolytic activity after isoelectric precipitation at different pH-values of (a) amniotic fluid + saline, (b) serum + saline, and (c) amniotic fluid + serum (equal volumes).

Abscissa: pH.
Ordinate: Fibrinolytic activity.

likewise produced an increase in fibrinolytic activity in guinea-pig serum after isoelectric precipitation. This reaction depended on pH (Fig. 5). None of three different samples investigated showed any spontaneous activity after precipitation, and even addition of a plasminogen activator (urokinase, kindly supplied by the Leo Pharmaceutical Co., Copenhagen) to samples precipitated at various pH-values produced no protease activity. This result indicates that plasminogen was absent, or present only in very small amounts, which is in accordance with the findings of Albrechtsen & Trolle (1955).

Saliva (mixed oral secretion) produced effect on serum (Fig. 6) comparable to th of the other fluids. Maximum fibrinoly activity was observed after precipitation pH 5.4—5.8. Two samples of saliva we used in our experiments and none of the produced lysis when directly applied to t fibrin plates. Albrechtsen & Thaysen (195 found that saliva contained no plasminog but that a plasminogen activator produci slight activity on normal fibrin was prese

Hyaluronic acid. Since some of the bo fluids investigated above are known to co tain hyaluronic acid, it is noteworthy th this substance (resembling other acid po saccharides) produced a pronounced eff similar to that of the body fluids (Fig. cf. Olesen (1959 b).

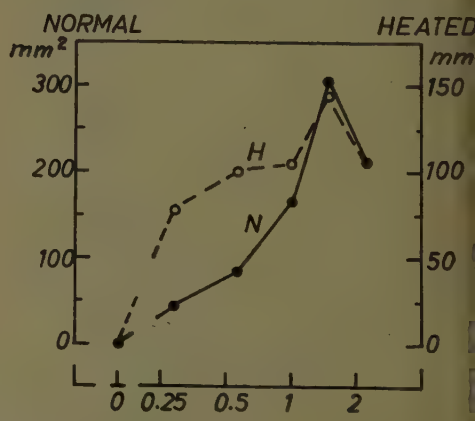


Fig. 6. Fibrinolytic activity after isoelectric p cipitation at pH 5.4 of guinea-pig serum with addition of saliva. Saliva, directly applied, produ no lysis.

Abscissa: ml of saliva per ml of serum (logarith scale).

Ordinate: Fibrinolytic activity. Different scales activity on normal and heated fibrin, (N) and (respectively.

DISCUSSION

he experiments demonstrate that preci-
tes prepared from mixtures of guinea-
serum with body fluids are more fibri-
tically active than those obtained after
rate precipitation of the serum and the
ective body fluid. This relation is espe-
y pronounced when the precipitation is
ormed at pH about 5.4. These observa-
s indicate an interaction of serum and
y fluid enhancing the formation of fibri-
tic activity. This effect of the body
ds is apparently similar to the activating
ct produced in guinea-pig serum by acid
saccharides, e. g. hyaluronic acid, and
other anionic polyelectrolytes (Olesen,
9 b, c).

hough the sample of hydarthron fluid
wed rather high spontaneous fibrinolytic
vity, the fact that the other fluids showed
e or no such fibrinolytic activity, taken
ther with the lack of plasminogen in
e of them (amniotic fluid, saliva), sug-
s that the fibrinolytic activity obtained
n mixtures of serum with a body fluid
erived mainly from the fibrinolytic system
he serum. This view is supported by the
larity of this reaction with the activation
duced in serum by anionic compounds,
e these do not constitute a source of
nolytic enzymes or precursors.

nionic compounds, as well as the body
ds investigated, produce the highest
nolytic activity when the mixtures with
ea-pig serum are precipitated at slightly
reaction. Protamine and cationic deter-
s, on the other hand, produce fibrino-
activity by isoelectric precipitation of
m at neutral reaction (Olesen, 1960).

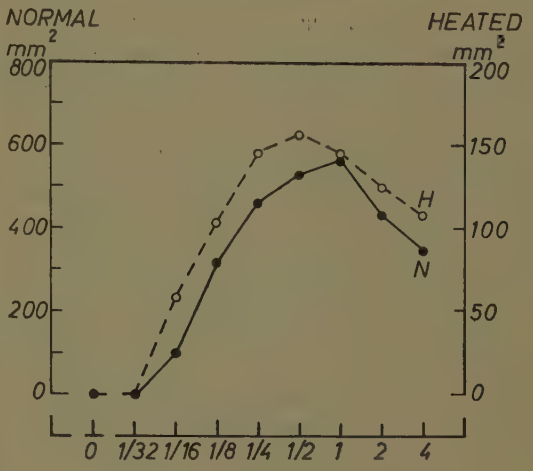


Fig. 7. Fibrinolytic activity of guinea-pig serum precipitated at pH 5.4 in the presence of hyaluronic acid (sodium salt).

Abscissa: mg sodium hyaluronate per ml of serum (logarithmic scale).

Ordinate: Fibrinolytic activity. Different scales for activity on normal and heated fibrin, (N) and (H), respectively.

It is therefore probable that the activating effect of the body fluids is caused by the presence of anionic agents such as hyaluronic acid or structurally related compounds.

The activating effect of acid polysaccharides has recently been confirmed by Serafini-Cessi (1959 a), who reported that hyaluronic acid as well as chondroitin sulphate causes precipitation of a fibrinolytic protein from guinea-pig serum at pH 5.2. An inflammatory reaction, experimentally induced intraperitoneally in guinea-pigs, had a similar effect on the precipitation of fibrinolytic agents from serum, probably brought about by an increased content of hyaluronic acid in serum (Serafini-Cessi 1959 b).

Guinea-pig serum has been used in the present investigation as a suitable indicator for the type of fibrinolysis activation caused by peptone, by acid polysaccharides, and by other anionic agents (Olesen 1959 a, b, c). Experiments with a purified peptone preparation (Astrup & Olesen 1957, Olesen 1959 a) indicated that the formation of fibrinolytic activity depends on the presence in guinea-pig serum of a plasminogen-activator system different from the 'pro-activator' system (Müllertz & Lassen 1953) which is involved in the production of fibrinolytic activity by streptokinase.

Human serum treated with 'purified peptone', was fibrinolytically inactive (Olesen 1959 a), but it has been demonstrated in other experiments (to be published), that human *plasma,* under similar conditions, regularly produces fibrinolytic activity. Thus, the prerequisites for a fibrinolysis producing interaction of the type here described appear to be available also in the human organism.

Halse (1948) reported that ascitic fluid, pleural effusions, hydrocele fluid, joint fluid, and cerebrospinal fluid accelerated the lysis of human plasma clots *in vitro* in experiments considered to approach physiological conditions. Halse's observations could possibly depend on interactions of the same type as those leading to a release of fibrinolytic activity in guinea-pig serum. It appears likely that the activating properties of the body fluids, as demonstrated in this paper, under certain conditions can contribute to fibrinolytic phenomena in the organism, *e. g.* the dissolution of fibrin in a hemarthron or a hematocele. The significance of pH for the activation in our experiment suggests tha local shift of pH could be of importance.

With reference to our observation of activating effect of amniotic fluid, it may recalled that abnormally high fibrinol activity of the circulating blood has b demonstrated in a case of amniotic fluid fusion (Albrechtsen, Storm & Trolle 195

The activating effect of saliva, in addit to the content of plasminogen activator pro-activator reported by Albrechtsen Thaysen (1955), could be of aid in keep free passage in the ducts of the saliv glands.

SUMMARY

Some human body fluids, compris fluids from a hydrocele, a hydarthron an hygroma, as well as amniotic fluid and sal enhance the formation of fibrinolytic acti in guinea-pig serum submitted to isoelec precipitation at slightly acid reaction.

This effect resembles the effect produ by acid polysaccharides, *e. g.* hyalur acid, and is probably caused by compou of a similar type.

Some possible implications concerning activation of fibrinolysis in the organism considered.

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URINARY ELIMINATION OF BROMSULFALEIN IN MAN

By K. WINKLER

From the Medical Dept. B. and the Cardiologic Laboratory of Medical dept. B., Rigshospitalet, Copenhagen, Denmark

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The major part of intravenously injected bromsulfalein (Sulfobromophthalein) is removed by the liver, but a small part can be recovered from the urine as demonstrated by Rosenthal & White (1924), who introduced the bromsulfalein retention test as a measure of hepatic function. It is the aim of the present work to assess the magnitude of the urinary elimination of bromsulfalein and the effect thereof on the total elimination from the blood in normal individuals and in patients with liver disease.

METHODS

Bromsulfalein (Hynson, Westcott & Dunning) was administered intravenously either as a single injection (5 mg/kg body weight) given in the course of a few seconds, or as an infusion by a motor-driven, calibrated syringe (2—9 mg/minute), in most cases preceeded by a "priming dose" (150—350 mg). Arterial or — in some cases — venous blood was drawn from an indwelling needle at intervals of 5—10 minutes.

With the exception of the cases in Table II b the urine was collected through a Foley-catheter. After evacuation the bladder was flushed twice with 20 ml of water and the total amount of fluid removed was mixed.

On direct measurement of bromsulfalein in the urine, the urinary blank density cannot be regarded, as it often amounts to the magnitude the density of the bromsulfalein present.

Determination of bromsulfalein in the urine made by a method identical with that employed plasma, using Gaebler's method (1945) for bl correction. The blank factor (*i. e.* the relation blank optical densities at 580 and 620 nm) determined on 44 samples of urine not contain bromsulfalein and was found identical with tha plasma (average 1.22, s. d. 0.20).

The absorbtion spectrum of bromsulfalein solved in urine is identical with that in plas The average standard deviation of the analyt procedure for urine, as calculated from 80 dupli determinations in the concentration interval 0- mg/100 ml was 0.05 mg/100 ml.

The urinary clearance was calculated as amount excreted in the urine per minute div by the mean plasma concentration in the urin sampling period. The plasma concentration obtained by graphical interpolation on the b elimination curves using a four minute displ ment for the time delay in the kidneys. After si injections with great decrements in concentra semilogarithmically drawn curves were used this interpolation. The amount of bromsulfa eliminated from the plasma was determined multiplying the concentration change at a g period by the plasma volume, assumed to be 5 cent of body weight (Bradley, Ingelfinger, Bra & Curry, 1945).

MATERIAL

The patients were examined recumbent in the rning and in the post-absorbtive state. No water s given during the experiments. The cases with reased elimination of bromsulfalein were pre-inantly patient with cirrhosis of the liver. No es with albuminuria were found, and the serum a was normal except in 3 of the cases with liver hosis (maximal value 67 mg/100 ml). In most eriments galactose was given simultaneously determination of the hepatic galactose eli-ation.

The following types of experiments were per-ned.

. Infusion of bromsulfalein was given to 24 mal individuals and 8 cases with liver disease. e sampling of urine started averagely 50 min-s after the onset of the infusion and continued 100 minutes with averagely 3 sampling periods. e plasma concentration usually rose slowly dur-the experimental period as is often found during longed bromsulfalein infusions. In 7 of the cases g. 1 and 2) the concentration was made to rise infusion of greater amounts (average rise 125 cent (58—218 per cent). In 4 of these cases the nary elimination was followed for 45 minutes r discontinuation of the infusion.

. Single injections of bromsulfalein with frac-ated urinary sampling were made in 8 normal es and 21 cases with liver disease.

n 65 further patients the urine was voided some e after the last blood sample had been secured. s group has been divided in cases with normal msulfalein elimination (45 minutes retention w 5 per cent), slight (5—20 per cent) or pro-nced (more than 20 per cent) impairment of the msulfalein elimination from the blood.

RESULTS

Infusion experiments.

Fig. 1 shows experiments with great rises plasma concentration after infusion of msulfalein to seven normal individuals. nerally the amount of bromsulfalein eli-

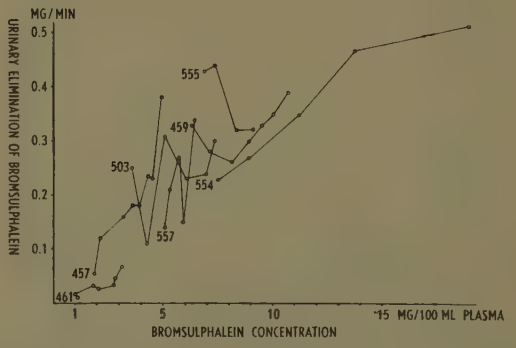

Fig. 1. Urinary elimination of bromsulfalein releated to plasma concentration in experiments with great rises in plasma concentration (7 normal indivuals).

minated in the urine per minute rises proportionally with increasing plasma concentrations. A constant urinary clearance is thus found with wide variations of the blood concentrations as seen in Fig. 2, where the clearance values throughout the experiment are shown. The 4 experiments with comparison of clearance at rising and falling plasma concentrations, not entered in the figure, showed slightly smaller figures for urinary clearance on the rising part of the plasma curve (clearance on rising concentration: 5.2, 4.5, 5.3, 3.7 ml/minute; falling concentration: ω 5.4, 5.1, 5.9, 6.4 ml/minute).

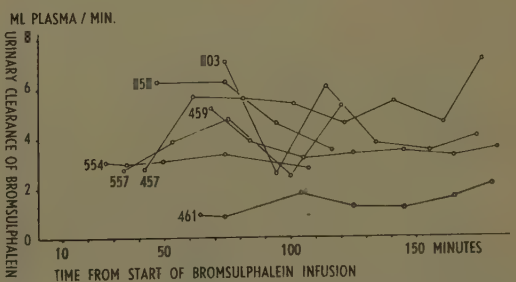

Fig. 2. Urinary clearance of bromsulfalein during infusion om bromsulfalein in the experiments of Fig. 1.

Fig. 3. Urinary elimination of bromsulfalein related to plasma concentration in normals and patients with liver disease, during infusion.

The experiments comprising normals and patients with liver disease with smaller rises in bromsulfalein concentration during infusion are depicted in Fig. 3. The average urinary clearances of the cases in Figs. 1 and 3 were not different, and the total material is shown in Table I. It is seen that the urinary clearance is about 5 ml per minute in the normal and pathologic cases, but that the urinary elimination in per cent of total elimination in the pathologic cases exceeds that of the normals, being averagely 5 times greater. The average plasma concentration in the two groups is essentially identical.

B. *Single injection experiments*

The single injection experiments with bladder catheterisation are summarized in Table II a. During the first 20 minutes, when the plasma concentration is decreasing rapidly, a very small part of the eliminated bromsulfalein appears in the urine in the normal cases and the urinary elimination in per cent of total elimination increases considerably throughout the experiment. The same phenomenom is also evident thou not so pronounced in the cases with liv disease. The experiments in which bladd catheterisation was not performed (Tab II b), show total urinary elimination of t same magnitude as that of the subje catheterized. With more pronounced impa ment of hepatic bromsulfalein removal, t urinary elimination in per cent of total e mination augments.

DISCUSSION

The material shows that the urinary e mination of bromsulfalein is small compar to the hepatic removal in normal individua The figures for renal elimination report here are in accordance with those stated the literature in animals (Werner & H wath 1952, Brauer, Pessoti & Krebs 19 Heinemann, Smythe & Marks 1953), and humans (Kessel 1956, Bradley *et al.* 19 Ingelfinger, Bradley, Mendeloff & Cran 1948).

The renal bromsulfalein excretion will i influence the result of a retention test sig ficantly in normals, and it will probably diminish the clinical usefulness of the test pathologic conditions (Norcross & Wh 1951). It is not likely that the normal re contribution to total elimination can be trac in the elimination curves from the blood, a 2 per cent change in plasma concentrat caused by renal elimination will be on borderline of analytical significance.

During infusion of bromsulfalein patients with decreased elimination of bro sulfalein, as much as 25 per cent of bromsulfalein given may be found in urine, and a correction for urinary loss t

ns necessary. This happens during deter-
ations of total plasma clearance in liver
eases. The same applies to determinations
he hepatic blood flow by Bradley's meth-
(1945), where significant urinary loss
es falsely high blood flow values.
As it is found that the urinary clearance
ndependent of the plasma concentration,
least below 10 mg/100 ml, a correction
be applied by determination of the aver-
elearance during the experimental period.
is value multiplied by the average plasma
centration in the intervals used for cal-
culation of the individual flow values by Bradley's method gives the correction for urinary loss.

The correction is of special importance if high plasma concentrations of bromsulfalein are used, as in determinations of the maximal bromsulfalein removal of the liver. Mason, Hawley & Smith (1948), using this method in dogs, found a renal elimination of 1—4 per cent of the amount infused at concentrations of 5—15 mg/100 ml, and stated that if still higher concentrations were used, the urinary excretion increased considerably.

Table I. *Urinary clearance of bromsulfalein during infusion.*

	Number of cases	Average amount infused mg/min.	Average urinary elimination		Average renal clearance	
			Per cent of total	S. D.	ml/min.	S. D.
rmals	24	8.75	1.9	1.0	4.6	1.8
er disease	8	2.20	9.3	8.5	4.8	2.5

Table II. *Urinary elimination of bromsulfalein during single injection.*

	Number of cases	Mean 45 min. retention, %	Amount injected mg.	Average sampling period. min.	Urinary elimination mg	% total plasma elimination
				a. bladder catheterisation.		
rmals	8	1.9	310	0—28	0.9	0.3
				28—49	1.7	24.6
				49—82	1.2	106.8
er disease	21	40	333	0—27	2.0	1.2
				27—49	5.2	16.6
				49—87	6.9	20.2
				b. without bladder catheterisation.		
rmals	19	2.5	310	0—98		1.3*
er disease	23	14	329	0—116		2.1*
»	23	34	311	0—104		4.7*

*per cent of amount injected.

Verschure (1952) found only 0.1—2 per cent urinary excretion, but was presumably working at rather small blood concentrations because of his short-lasting experiments. We have found (unpublished), that the total plasma clearance of bromsulfalein decreases considerably with rising blood levels during infusion. Thus, on constant renal clearance, the renal fraction of totally removed bromsulfalein will be relatively more important as the plasma concentration rises.

The delayed renal elimination found in the experiments with single injections of bromsulfalein makes correction for urinary loss impossible. The cause of the phenomenon might be storage of bromsulfalein in the kidney cells (Homer Smith 1950), or mixing processes in the urinary collecting system (Brun, Hilden & Raaschou 1949). The infusion experiments with comparison of renal clearance at rising and falling plasma concentrations indicate that the delay-phenomenon does not play a great role during the experimental conditions, where infusion of bromsulfalein are usually performed. The delay in urinary excretion of bromsulfalein observed during single injections is much greater than the time delay due to mixing processes in the kidney (Mc Swiney & Wardener 1950) even with the rather small diureses met with in the present work, where no extra water was supplied. The occurrence of bromsulfalein metabolites in the blood some time after an injection (Carbone, Grodsby & Hjelle 1959) might explain the delay in the urine, if these substances have greater clearance than the native bromsulfalein.

The renal mechanism for the elimination of bromsulfalein cannot be elucidated from the present experiments. As no measurable quantity of bromsulfalein can be found fr in plasma by dialysis (bromsulfalein plasmaprotein bound, most probably serum albumin (Bradley *et al.* 1945, Pezo 1953)), a clearance of 5 ml plasma per n nute indicates tubular secretion. The val for renal bromsulfalein clearance is mu greater than can be accounted for by re elimination of plasma proteins, even in cas with gross albuminuria.

SUMMARY

The renal clearance of bromsulfalein h been found to about 5 ml plasma per minu during infusions to normal subject and patients with different degrees of liv disease.

Normally, 2 per cent of the bromsulfale given could be found in the urine, in cas with liver diseases it amounted to average 9 per cent. When bromsulfalein infusions a used for determination of total plasm clearance as indicator of the hepatic functi or the hepatic blood flow in patients wi diminished bromsulfalein removal, a corre tion for urinary loss must be applied. Duri single injections of bromsulfalein a gre time-delay was found in the urinary elim nation making exact correction for the elim nation impossible.

Bromsulfalein is eliminated in the kidne by tubular secretion.

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THE DETERMINATION OF THIAMINE AND THIAMINE PHOSPHATES IN BLOOD AND TISSUES BY THE THIOCHROME METHOD

By H. N. HAUGEN

From The Institute for Medical Biochemistry and Physiology, University of Oslo, Oslo, Norway

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Since the thiochrome method was introduced for the determination of thiamine (Jansen 1936), this method has been used also for the estimation of thiamine in blood and tissues. When the thiamine concentration is determined in these fluids, protein precipitation is necessary, followed by extraction of thiamine, and because thiamine occurs both as free thiamine and in the phosphorylated form, hydrolysis is also necessary.

Metaphosphoric acid was used for protein precipitation both by Friedemann & Kmieciak (1943), who determined thiamine in blood, and by Greenberg & Rinehart (1945), who also estimated tissue thiamine, and by Hjarde (1950), who studied thiamine concentration in different organs. A simplification was the adoption of trichloracetic acid as a protein precipitant (Burch, Bessey, Love & Lowry 1952), originally used for thiamine determination by Hennessy & Cerecedo (1939). Most investigators used diastase or clarase for hydrolysis of phosphorylated thiamine (Friedemann & Kmieciak 1943, Greenberg & Rinehart 1945, Foa *et al.* 1947, Hjarde 1950). Burch *et al.* (1952), however, found a phosphatase prepared from clarase inconvenient and used a acid phosphatase prepared from huma seminal fluid.

The non-specific fluorescent compoun present in all biological fluids or develop during oxidation of thiamine to thiochrom have been determined, or excluded, in diffe ent ways. Both Friedemann & Kmieci (1943), Greenberg & Rinehart (1945), a Foa *et al.* (1947) used adsorption-eluti procedures. Hjarde (1950) used an alka treated sample (where thiamine is destroye as a blank, whereas Burch *et al.* (1952 preferred to destroy the thiochrome by ultr violet light and to use the remaining fluo escence as a blank.

In a recent publication (Haugen 196 benzenesulphonyl chloride was found sat factory for preparation of the urine bla Because this method was reliable, sensiti and relatively rapid, an attempt was made use benzenesulphonyl chloride also determination of free and phosphorylat thiamine in blood and tissues. The purpo of this paper is to describe the method a to report the values obtained.

METHODS

paration of protein-free extracts.

Human blood. Ten ml of heparinized blood were vered slowly into a 200 ml Erlenmeyer flask aining 46 ml of a 0.3 N solution of trichlor- ic acid. The flask was stirred continuously ng the addition of blood and left at room tempe- re for half an hour. Following centrifugation 500 r.p.m. for 15 minutes, a clear supernatant obtained for determination of free and phos- rylated thiamine.

Rat blood. Two ml of rat blood, obtained by ipitation, and 9.2 ml of trichloracetic acid were l. After centrifugation, the supernatant was ted with four volumes of 0.246 N trichloracetic to obtain a sufficient quantity of fluid for rmination of free and phosphorylated thiamine s concentration of trichloracetic acid was used eep the diluted supernatant at the same pH as undiluted supernatant).

Tissues. Five ml of a 0.3 N solution of trichlor- ic acid were pipetted into a Potter-Elvehjem ogenizer. A piece of tissue, *e.g.* rat liver ghing 1—2 grams, or both rat kidneys, was sferred to the homogenizer. The necessary vol- of trichloracetic acid solution to give 4 vol- s of acid for each gram of tissue was added, the tissue completely homogenized within 1—2 utes. After centrifugation at 15 000 r.p.m. at 2° C 10 minutes, a clear, slightly yellow fluor- nt supernatant was obtained. For determination free thiamine, the supernatant was diluted ten s with 0.246 N trichloracetic acid; for deter- ation of phosphorylated thiamine in kidney or r homogenates, a dilution of 1:500 with 0.246 N hloracetic acid was found convenient.

ermination of the concentration of free thiamine blood and tissues.

Ten ml of the clear blood supernatant or the 0 dilution of tissue supernatant were trans- ed to a burette and from this slowly added (in seconds) with agitation to a mixture of 5 ml 40 per cent solution of sodium hydroxide and ml of a 2 per cent solution of potassium ferri- ide. The agitation was continued for another econds, whereupon 2 drops of a 3 per cent sol- n of hydrogen peroxide were added to destroy possible excess of potassium ferricyanide. Ten ml of redestilled isobutanol were then added, the mixture shaken for half a minute, and transferred to a centrifuge tube. After centrifugation for 2 minutes, the isobutanol layer was pipetted off. If the fluorescence of the isobutanol layer was not read immediately, the extract was kept in the dark and recentrifuged before determination of the fluorescence.

The blank was treated in the following way: To ten ml of the supernatant or the 1:10 dilution of tissue extract were added 6 drops of a 40 per cent solution of sodium hydroxide and one drop of benzenesulphonyl chloride. After vigorous stirring for 45 seconds, the mixture was transferred to a burette and treated in the same way as the sample.

For calculation of the free thiamine concentration, two samples of a thiamine standard, usually 1 µg/100 ml, were treated in the same way as the sample and the blank respectively.

(For determination of free thiamine in plasma and blood cells, a modification of the protein precipitation proposed by Burch *et al.* (1952) was used. For plasma, 10 ml were precipitated with 55 ml of 0.3 N trichloracetic acid. For blood cells, 5 ml were added to 27.5 ml of 0.85 per cent saline and then precipitated with 27.5 ml of 0.6 N trichloracetic acid.)

Determination of the concentration of phosphorylated thiamine in blood and tissues.

The hydrolysis of thiamine phosphates was performed according to a modification of the method of Burch *et al.* (1952): Twenty ml of the blood supernatant or of the 1:500 dilution of tissue extract were pipetted into a 30 ml reagent tube, and 2.5 ml of a 4 M solution of potassium acetate were added to give a pH of about 4.6. The hydrolysis was accomplished by the addition of a sufficient quantity of acid phosphatase, prepared according to Burch *et al.* (1952) (0.4 ml was found satisfactory). The tubes were covered with aluminum foil to prevent dust contamination and left overnight in a water bath at 37° C. On the next day 10 ml of the solution were treated as the supernatant and the extract above while another 10 ml were used as the blank. A standard solution of thiamine pyrophosphate, usually 1 µg/100 ml, was run simultane-

ously for calculation of the total thiamine content of the blood and tissues.

(In this study a thiamine pyrophosphate standard was used for comparison, and the values obtained in blood and tissues referred to as "total thiamine". This designation includes both free thiamine and thiamine obtained by hydrolysis of phosphorylated thiamine.)

For reading of the fluorescence, a Farrand Optical Co., Inc. spectrofluorometer was used; the excitation was done at a wavelength of 365 mμ, the fluorescence read at 435 mμ.

Before the results are presented, some steps of importance for the determination of free and total thiamine should be discussed.

The concentration of potassium ferricyanide.

Due to the well known quenching effect of potassium ferricyanide, too much of this oxidizing reagent should be avoided. This is of particular importance when the small amount of free thiamine in the blood is determined. Table I shows the results of using increasing amounts of potassium ferricyanide for the oxidation of thiamine in a blood supernatant. Increasing the amount of a 2 per cent solution of potassium ferricyanide from 0.05 to 0.30 ml reduced the fluorometric reading from 84 to 31. However, when the thiamine content of the supernatant was raised by adding 0.5 μg of thiamine hydrochloride to 70 ml of the supernatant, 0.05 ml of potassium ferricyanide was insufficient for complete oxidation of thiamine to thiochome (Table II); a potassium ferricyanide amount of 0.10 ml gave

Table I. *The influence of increasing amounts of potassium ferricyanide upon the fluorescence of trichloracetic acid blood supernatants.*

Amount of 2 per cent potassium ferricyanide used for oxidation (ml)	Fluorometric reading
0.05	84
0.10	58
0.20	33
0.25	30
0.30	31

Table II. *The influence of increasi amounts of potassium ferricyanide upon t fluorescence of trichloracetic acid blood s pernatants when thiamine is added.*

Amount of 2 per cent potassium ferricyanide used for oxidation (ml)	Fluorometric reading
0.05	150
0.10	165
0.20	136
0.25	118
0.30	117

maximum fluorescence whereas larger amou gave lower fluorescence.

An identical amount of potassium ferricyani is sufficient for the determination of thiamine tissue homogenates, probably because other reduci substances are present in low concentration due the dilution. For determination of total thiamine blood and homogenates, 0.2 ml of a 2 per ce solution was used.

The enzymatic conversion of phosphorylated thiamine to thiamine.

The accuracy of determining total thiamine blood and tissues depends upon complete hydroly to thiamine. The effectiveness of the procedure w tested by submitting different concentrations thiamine pyrophosphate to hydrolysis. A net g vanometer deflection of 3.12 units per 0.02 micr moles of hydrolyzed thiamine pyrophosphate w obtained while a corresponding concentration thiamine hydrochloride gave a deflection of 3. units. Thus, the hydrolysis was considered comple

Stability of the trichloracetic acid supernatants.

It was accidentally found that an analysis peated on the next day gave a higher value th the original one, and this prompted a study of t stability of the trichloracetic acid supernatan Supernatants were left in the refrigator at 4° for one week to 26 days and re-examined. T results (Table III) show that an increase in t fluorometric reading occurred during storage.

ble III. *The effect of storage upon the rometric readings of trichloracetic acid upernatants calculated as total thiamine.*

uper-ant No.	Original total thiamine values (μg/100 ml)	Values upon reexamination	Stored for (days)
1	3.15	4.75	17
2	1.53*	3.57	16
3	2.59	5.31	7
4	3.40	7.52	24
5	2.77	4.51	23
6	4.68	8.45	26

blood from a diabetic patient

RESULTS

covery experiments and duplicate erminations.

Table IV shows the recovery of added mine pyrophosphate in 8 experiments. e recovery varied between 93.5 and 111.8 cent with an average of 104.7 per cent. e difference between duplicates in 31 de-minations ranged from 0 to 9.2 per cent, average being 3.5 per cent.

ble IV. *Recovery of added thiamine pyrophosphate to blood.*

od o.	Total thiamine concentration (μg/100 ml)	Thiamine pyrophosphate added (μg/100 ml)	Total thiamine found (μg/100 ml)	Recovery (per cent)
	5.07	6.00	11.02	99.2
	4.63	4.00	8.73	102.5
	4.63	6.00	11.04	106.8
	4.14	4.00	8.41	106.8
	4.14	6.00	9.74	93.5
	5.04	4.00	9.51	111.8
	3.31	4.00	7.55	106.0
	4.04	5.00	9.60	111.0
			Average recovery	104.7

Free thiamine of whole blood.

In 11 healthy subjects (medical students and laboratory associates) the concentration of free thiamine in the postabsorptive state ranged from 0.65 to 1.14 μg/100 ml of blood. After thiamine administration a temporary increase of the value was observed. Thus, in a healthy individual given 5 mg of thiamine hydrochloride for 5 days, the concentration of free thiamine in the postabsorptive state increased from 0.65 to 1.07 μg/100 ml of blood; on the following day, when thiamine was withdrawn, a value of 0.89 μg/100 ml was found.

Table V. *Total thiamine concentration of the blood on consecutive days in 4 individuals.*

Subject No.	Total thiamine concentration of blood (μg/100 ml)			
1	6.13	6.24	6.10	
2	3.21	2.95	2.48	3.07
3	4.46	4.35	4.26	
4	7.08	6.88		

Total thiamine of whole blood.

In healthy individuals the values showed a large variation, the lowest value being 2.48 μg/100 ml, the highest 10.77 μg/100 ml. Most of the values ranged between 4 and 7 μg/100 ml, the average in 43 determinations being 5.28 μg/100 ml. The day to day fluctuation was remarkably low, as will be seen from Table V. When blood examinations were repeated after a prolonged time interval, larger variations were found. In one healthy individual, levels of 7.08 and 6.88 μg/100 ml were found on two consecutive days; two months later, however, values of

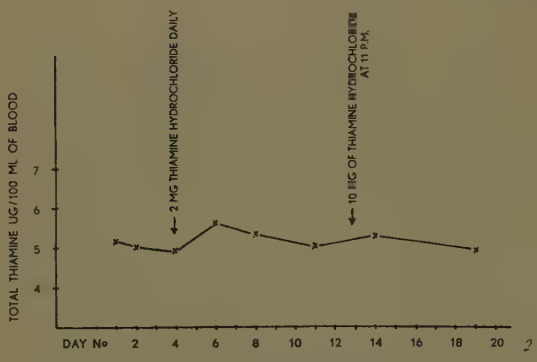

Fig. 1. Influence of administering thiamine orally to a healthy individual.

5 μg/100 ml were obtained. The administration of thiamine orally seemed to play only a minor role. In one individual the levels on a regular diet were about 5 μg/100 ml (Fig. 1). After 2 mg of thiamine hydr chloride daily for two days the total thiami levels were 5.68 μg/100 ml, 5.35 μg/100 and 5.06 μg/100 ml during continued adm nistration of thiamine. Upon administrati of a larger dose (10 mg) no significant i crease was seen.

The concentration of free and total thiami in rat blood, liver and kidney.

The values obtained in 11 rats will be se from Table VI. In two rats the concentr tion of free thiamine in the blood was 0. and 1.82 μg/100 ml. The total thiamine co centration of the blood showed values mu higher than those of humans. The avera

Table VI. *Concentration of free and total thiamine in rat liver, kidney, and blood.*

Rat No	Liver		Kidney		Blood	
	Total thiamine (μg/gram of wet tissue)	Free thiamine (μg/gram of wet tissue)	Total thiamine (μg/gram of wet tissue)	Free thiamine (μg/gram of wet tissue)	Free thiamine (μg/100 ml)	Total thiamine (μg/100 ml)
			Non-fasted rats			
1	15.2	0.31	8.4	0.18		16.9
2	17.3	0.41				
3	13.8	0.39	11.3	0.60		34.0
4	14.5	0.18	12.6	0.69		35.6
5	21.3	0.52	13.8	0.74		14.0
6	15.8	0.17	15.2	0.57		32.9
7	13.1	0.22	9.7	0.35		
Average	15.9	0.31	11.8	0.52		20.7
			Rats fasted for 24 hours			
8	12.9	0.22	10.8	0.31		26.3
9	13.6	0.18	8.2	0.24		19.4
10	12.3	0.14	8.3	0.15	1.82	20.5
11	10.7	0.12	8.3	0.15	0.84	19.1
Average	12.4	0.17	8.9	0.21		21.3

centration of non-fasted and fasted rats re identical, but the values varied widely. e concentration of free thiamine in liver l kidney was low, 0.12 to 0.74 μg/gram wet tissue. The total thiamine concentra- n in the liver and kidney varied between and 21.3 μg/gram, the liver values in all es being higher than the kidney concen- tions.

DISCUSSION

Baker, Pasher, Frank, Hutner, Aaronson Sobotka (1959), using a microbiological thod for the determination of thiamine, tioned against the use of trichloracetic l for protein precipitation because it re- ted in low thiamine values. The present dy does not confirm this statement. The overy is satisfactory, and the values ob- ned are in agreement with those reported others (see below). Also Burch *et al.* 952) found the trichloracetic acid pre- itation satisfactory. On the other hand, hloracetic acid supernatants showed in- ase of fluorescence when stored over a longed time, the values being consider- y higher upon re-examination. Also Dubé, nson, Yü & Storvick (1952) observed her fluorometric readings of samples ich had been kept frozen. Therefore, tri- oracetic acid supernatant should be ana- ed immediately after centrifugation.

Non-thiamine fluorescent substances are sent in all biological fluids, and non-thio- ome fluorescent compounds develop dur- the oxidation of thiamine to thiochrome. erefore, a correct blank is necessary. For eous solutions of thiamine and for urine, zenesulphonyl chloride was found satisfactory (Haugen 1960). The present study indicates that benzenesulphonyl chloride can be used also for the determination of thiamine in blood and tissue.

The good recovery is an indication that the method is reliable as pointed out above. Furthermore, the values conform well with values obtained in other studies. In human blood or plasma the free thiamine level is very low, and data obtained by methods comparable with the present one are not available. However, in microbiological methods a concentration of about 1 μg/100 ml is usually reported. The values obtained are in agreement with these reports.

The values for total thiamine concentration of the blood also conform well with the values obtained by the thiochrome method in other studies. Burch *et al.* (1952) reported values of 4.3 to 5.2 μg/100 ml of blood. In Greenberg & Rinehart's (1945) study, 5.0 to 10.0 μg/100 ml were found. Friedemann & Kmieciak's (1943) findings showed a wider range, 3 to 11.2 μg/100 ml, a range comparable to that obtained in the present study.

The concentration of free thiamine of the rat liver and kidney was lower than the values reported by others. Hjarde (1950) found a concentration of free thiamine of 1.46 μg/gram in the liver of a rat given 2 mg of thiamine daily for 14 days and sacrified 24 hours after the last injection. In the kidney 0.82 μg/gram was found. The higher free thiamine concentration in Hjarde's (1950) study may be due to the massive doses of thiamine administered prior to the examination. In the study of Foa, Weinstein, Smith & Greenberg (1952) much higher values were found in the liver, the average concentration in 11

normal rats being 1.9 μg with a range from 1.1 to 2.4 μg/gram. The reason for the discrepancy between the values of Foa *et al.* and those obtained in the present study is not obvious.

The finding of a higher concentration of free thiamine in the kidney than in the liver is unexplained. A higher concentration of total thiamine in the liver, however, was found also by Hjarde (1950) although his figures were somewhat lower. The high liver concentration of total thiamine may have relation to the multitude of enzymatic reactions in the liver requiring active thiamine as a co-factor or to the liver being a storage organ for thiamine.

SUMMARY

A reliable and relatively rapid method is described for the determination of free and total thiamine in blood and tissues, using the thiochrome method and, for determination of the blank, benzenesulphonyl chloride. The values obtained for blood agree well with those obtained by other thiochrome methods. The values for free thiamine in the liver and kidney are lower, for total thiamine higher, than those reported by other modifications of the thiochrome method. The reasons for the discrepancies are briefly discussed.

ADDENDUM

The values for free thiamine recently reported by Rindi & De Giuseppe (Rindi, G. & De Giuseppe, L.: Thiamine and its mono-, di, and triphosphoric esters content of normal rat tissues *in* Experientia 16, 447, 1960) conform well with the values obtained in the present study.

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EXCRETION OF THIAMINE IN DIABETES: UNRELATED TO GLYCOSURIA AND POLYURIA

By H. N. HAUGEN[1]

From Departments of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Massachusetts, U.S.A.

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Because of the similarity of diabetic peri-
ral neuropathy to the neuropathy seen in
amine deficiency, the urinary excretion of
amine in diabetes has received much at-
tion. Several studies have been performed,
the renal excretion of thiamine in dia-
es is still insufficiently known. The rea-
s for the reported differences are not ob-
us. However, the relationship between
cosuria and polyuria on one hand and
amine excretion on the other has received
y minor attention. The present study
s undertaken in an attempt to investigate
influence of glycosuria and polyuria
n the excretion of thiamine.

MATERIAL AND METHODS

Four diabetic individuals were studied, one of them twice on the same regimen. They were given a constant diet conforming with their usual food intake as to content of calories, carbohydrates, protein, and fat. The diet was not identical for all individuals because it was considered more important to give each patient a diet similar to his usual diet. No additional vitamins were given. Insulin was administered as required, but the control was not ideal because the patients had the juvenile type of diabetes. None of the patients showed any clinical evidence of vitamin deficiency.

The most important data regarding the patients will be seen from Table I.

The 24 hour urine samples were collected in brown bottles with toluene as a preservative. The urine was kept in the refrigerator until it was ana-

Table I. *The most important data regarding the patients' age, duration of diabetes, insulin requirement, complications and diet.*

tient	Age (Years)	Duration of diabetes (Years)	Insulin (units per day)	Complications	Calories	CHO (Gm)	Fat (Gm)	Protein (Gm)	Thiamine (μg)
A.	56	8	70	None	2042	202	92	101	1446
W.	20	11	60	Very early retinopathy	2373	208	117	111	1425
McG	22	14	31	Small hemorrh. left fundus. Tendon reflexes weak	2461	197	137	110	1219
P.	38	5	45	Slight proteinuria. Blood urea nitrogen 7 mg per 100 ml	2383	244	115	119	1323

[1] Present address: The Institute for Medical Chemistry and Physiology, University of Oslo, Oslo, Norway.

Table II. *Urinary volume, fasting blood glucose values, and daily excretion of creatinine, glucose, and thiamine in patient B.P.*

Day	Urinary volume (ml)	Blood glucose (mg/100 ml)	Creatinine in the urine (mg)	Glucose in the urine (Gm)	Thiamine in the urine (μg)
1	3,220	311		64	275
2	5,200	22	1279	175	290
3	3,630	325	1510	197.4	261
4	3,400	221	1566	139	265
5	3,260	350	1526	120	258
6	2,960	244	1098	93	271
7	4,160	324	1273	85	250
8	3,040	236	1107	76	290
9	3,620	288	1296	97	275
10	3,200	300	1290	38	256
11	3,500	161	1190	77	270
12	3,690	292	1345	91.5	300
13	2,420		1253	11.2	280
14	3,180	256	992	70	260
15	2,820	238	1187	147	255

lyzed. Analysis for thiamine was performed as soon as possible; if postponed for more than a day, hydrochloric acid was added to make the samples distinctly acid. The creatinine excretion was used as a control of complete urine collection.

Aliquots of the 24 hour urine were analyzed for thiamine by the thiochrome method, using benzenesulphonyl chloride as a blocking agent for determination of the blank (Haugen 1960). Blood glucose was measured by the method of Nelson (194 urinary true glucose by the method of Froesch Renold (1956), creatinine by the method of Bo nes & Taussky (1945).

RESULTS

Table II shows the results of a study patient B.P. (who was studied twice). T

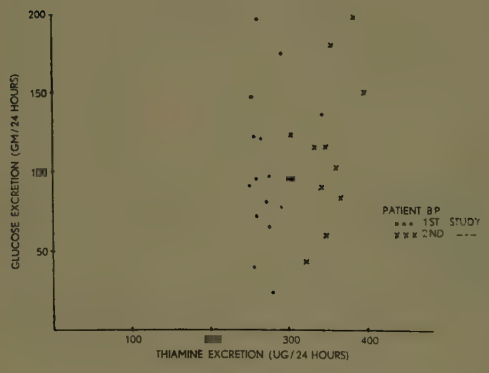


Fig. 1. Excretion of thiamine and glucose in patient B.P.

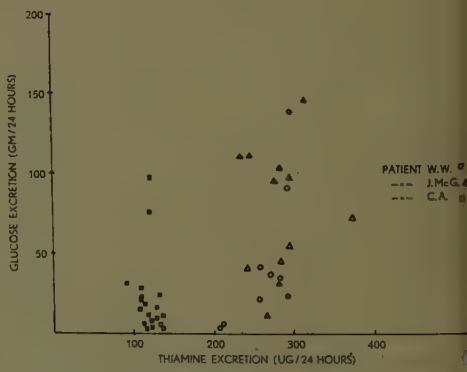
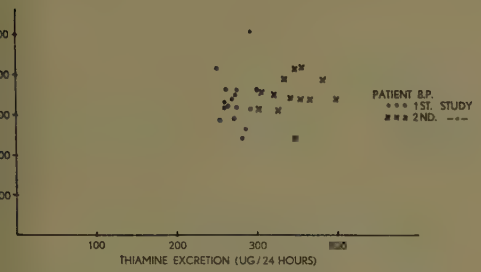


Fig. 2. Excretion of thiamine and glucose in pati W.W., J. McG., and C.A.



3. Thiamine excretion and urine flow in patient B.P.

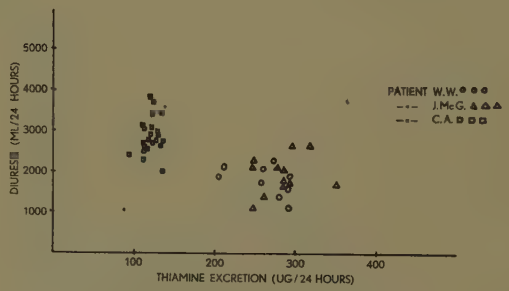


Fig. 4. Thiamine excretion and urine flow in patients W.W., J. McG., and C.A.

resis was high, mainly because this patient s used to a high fluid intake, but probably due to the large excretion of glucose. e blood glucose values were high, except one day when the patient suffered a brief ılin reaction. The glucose excretion varied ely, from 11.2 to almost 200 g per 24 rs. In contrast, the thiamine excretion rather constant: 250 to 300 μg per 24 rs. There was obviously no relationship ween the excretion of glucose and the ex- ion of thiamine. On day 13, when only 2 g of glucose were excreted, 280 μg of mine were eliminated; an almost iden- l amount was excreted on the day of ximal glycosuria. The relationship be- en glycosuria and thiamine excretion in patient is seen from Fig. 1. The cor- tion is obviously so poor that no stati- al calculation is necessary. The same lies to the other patients.

Table II shows that the excretion of thia- e in patient B. P. was uninfluenced by urinary volume. The thiamine excretory ues were almost identical on diureses of 0 and 2420 ml a day. The relationship ween urinary volume and thiamine excre- in all patients will be seen from Fig. 3 4. No correlation was found.

DISCUSSION

It is well known that the renal excretion of one solute may influence the excretion of other solutes. Shannon (1938) showed that the tubular reabsorption of xylose is completely blocked when the plasma level of glucose is raised to approximately 300 mg per cent. Daughaday & Larner (1954 a, b) demonstrated a renal tubular reabsorption mechanism for inositol, and found that high loads of glucose blocked this reabsorption. Bonsnes & Dana (1946) demonstrated a higher rate of uric acid excretion and therefore a higher uric acid clearance during intravenous infusion of hypertonic glucose; they assumed that glucose and uric acid might compete in some mechanism involved in the reabsorption processes. According to Homer Smith (1951), however, the higher uric acid clearance during glucose infusion is due to the osmotic diuresis induced by glucose.

The renal mechanism for thiamine excretion is insufficiently known. A low excretion rate is likely for two reasons: (1) thiamine is essential to the organism, and (2) low thiamine excretory values are found when the thiamine content of the diet decreases, both pointing to a low "clearance" and a

high reabsorption rate in the renal tubules. Since, during glycosuria, the glucose reabsorbing mechanism is more or less saturated, a relationship between the excretion of thiamine and the excretion of glucose in the urine wold not be unreasonable.

No such relationship between the daily excretion of glucose and of thiamine could be demonstrated in our diabetics. This might indicate that the glucose reabsorptive mechanism was not saturated, or, more likely, that glucose excretion does not influence the excretion of thiamine. A possible thiamine deficiency in diabetes is, therefore, not caused by glycosuria.

As to the influence of urinary volume upon thiamine excretion, Borson (1940) maintained that thiamine is a "threshold" substance that may be "washed out" by a large urine flow, and polyuria might therefore contribute to thiamine deficiency. In Wassmann's (1944) study polyuria (induced by high fluid intake) resulted in increased thiamine excretion in 4 of 5 individuals. On the other hand, Lowry & Hegstad (1945), from their study in alloxan diabetic rats, concluded that no "wash out" of thiamine had taken place. In the present study, thiamine excretion was independent of urinary volume in every patient. A fair conclusion is that thiamine excretion in diabetics is independent of urinary volume, and a possible loss of thiamine in diabetes is not due to polyuria.

SUMMARY

The urinary excretion of thiamine was studied in four diabetic individuals given a constant diet, in an attempt to determine the relationship between thiamine excretion on one hand, and glycosuria and polyuria on t other. No correlation was found between t daily excretion of thiamine and the daily e cretion of glucose, or the diuresis. It is co cluded that a possible thiamine deficiency diabetes is caused neither by glycosuria n by polyuria.

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ENAL THIAMINE EXCRETION IN THE DOG; INFLUENCE OF PARENTERAL ADMINISTRATION OF GLUCOSE

By H. N. HAUGEN
(with the technical assistance of Aud Vogt)

From The Institute for Medical Biochemistry and Physiology, University of Oslo, and The Institute for Experimental Medical Research, Ullevål Hospital, Oslo, Norway

In this paper three problems are discussed, (1) the normal renal excretion of thiamine (2) the excretion after parenteral administration, and (3) the excretion of thiamine during a heavy glucose load.

The normal values for thiamine clearance not known. In man the average daily cretion on a regular diet is about 100— μg, indicating a relatively low clearance e below). On the other hand, it is well own that thiamine administered parenter- is excreted very rapidly. In Wassmann's 44) study almost 1,000 μg were excreted ring the first four hours following the intramuscular injection of 5 mg of thiamine, l when an identical dose was administered avenously, 1,300 μg were excreted in four urs. This points to a high clearance after enteral administration. Thus, thiamine y be a substance reabsorbed in the tubules low plasma concentrations, but excreted through the tubular cells upon parenteral ministration.

The problem of thiamine excretion during cosuria is both of physiological and practical importance. It is an established fact that ious substances excreted in the urine may influence the excretion of other substances. The best known examples are the lowered tubular excretion of penicillin after the administration of carinamide (4′-carboxyphenylmethanesulfonanilide) and probenecid (p-(di-n-propylsulfamyl)-benzoic acid) and the increased urate excretion after administration of the same drugs. Furthermore, the tubular reabsorption of xylose (Shannon 1938) and inositol (Daughaday & Larner 1954 a, b) is depressed by heavy glucose administration, thus giving a high clearance of these substances. From a practical point of view the problem is interesting because of the discussion of thiamine deficiency in diabetes mellitus and its possible relationship to urinary "loss" of thiamine.

The present study was undertaken in an attempt to elucidate these problems.

MATERIAL AND METHODS

Two healthy mongrel dogs, Alma weighing 11 kg, and Ammy, 14 kg, were used. The dogs were fed cooked maize supplemented with cooked meat, the average daily protein intake being about 100 g. In addition cod liver oil and a mineral-vitamin preparation was given.

No food was given on the day of the experiment. No anesthesia was used. To secure a copious diuresis, about 500 ml of tap water was given through stomach tube 45 minutes before each experiment. Urine was collected by an indwelling Foley catheter. The bladder was rinsed twice before the experimental periods were started and also after each period in order to obtain complete urine collection. Blood samples were obtained in the middle of the experimental period from a needle introduced into the cephalic vein of the foreleg. The administration of glucose and/or thiamine was performed through another vein. Twenty to 30 minutes were allowed for establishment of equilibration of glucose and thiamine. The experimental periods lasted 10 to 15 minutes.

During the infusion of the priming solutions of thiamine the dogs showed slight restlessness. During the experimental periods, however, no adver signs were noted.

The amounts and concentrations of glucose a thiamine given will be seen from Table I—V.

Creatinine was determined according to t alkaline picrate method, modified after Laus (1951), glucose in blood and urine by the meth of Nelson (1944), modified by Somogyi (195 Thiamine was determined according to the thi chrome method (Haugen 1960 and 1961). In ma ing the clearance calculations, plasma thiamine w assumed to be completely diffusible.

RESULTS

The results are presented and described Table I—V.

Table I. *Experiment 1. Dog Alma. No additional thiamine was given before the experiment. Priming infusion of 10 grams of glucose was given before period 3, sustaining infusion of 600 mg/minute given during periods 3 and 4.*

	Control periods		Glucose periods	
	1	2	3	4
Plasma thiamine concentration (μg/100 ml)	0.48	0.34	0.34	0.34
Thiamine excreted (μg/minute)	0.03	0.02	0.01	0.02
Thiamine "clearance" (ml/minute)	6.4	5.9	3.3	7.1
Creatinine clearance (ml/minute)	45	51	49	49
Blood glucose concentration (mg/100 ml)			440	330
Glucose excreted (mg/minute)			100.4	15.2

The concentration of plasma thiamine was low throughout the experiment as was the excretion of thiamine. Also the "clearance" of thiamine was low. No change in thiamine excretion occurred during the infusion of glucose.

Table II. *Experiment 2. Dog Alma. Thiamine hydrochloride, 100 mg daily, was injected subcutaneously for four days before the experiment. Priming infusion of 2 mg of thiamine hydrochloride was administered before period 3, sustaining infusion of 10 μg/minute was given during periods 3—6. Priming infusion of 10 grams of glucose was given before period 5, sustaining infusion of 600 mg/minute was given during periods 5 and 6.*

	Control periods		Thiamine periods		Glucose + thiamine periods	
	1	2	3	4	5	6
Plasma thiamine concentration (μg/100 ml)	0.92	0.92	6.71	6.80	6.91	7.05
Thiamine excreted (μg/minute)	0.48	0.37	7.16	6.28	6.16	6.25
Thiamine "clearance" (ml/minute)	53	40	106	92	90	89
Creatinine clearance (ml/minute)	46	44	42	40	47	38
Blood glucose concentration (mg/100 ml)					431	436
Glucose excreted (mg/minute)					110	99

The concentration of plasma thiamine was higher than in Experiment 1. Also control excretion of thiamine was higher, and increased to 7.16 and 6.28 μg per minute, respectively, during the infusion of 10 μg per minute of thiamine hydrochloride. Both plasma thiamine concentration and thiamine excretion remained unchanged during the glucose infusion and excretion.

Table III. *Experiment 3 (performed 8 days after Experiment 2). Dog Alma. No additional thiamine was given before the experiment. Priming infusion of 4 mg of thiamine hydrochloride was administered before period 3, sustaining infusion of 20 μg per minute was administered during periods 3—6. Priming infusion of 10 grams of glucose was given before 5, sustaining infusion of 600 mg of glucose/minute was given during periods 5 and 6.*

	Control periods		Thiamine periods		Glucose + thiamine periods	
	1	2	3	4	5	6
Plasma thiamine concentration (μg/100 ml)	0.79	0.79	14.69	11.67	5.53	6.29
Thiamine excreted (μg/minute)	0.15	0.19	38.86	30.50	12.07	10.00
Thiamine "clearance" (ml/minute)	19	24	265	261	218	159
Creatinine clearance (ml/minute)	51	62	54	52	54	55
Blood glucose (mg/100 ml)					260	260
Glucose excreted (mg/minute)					34.7	14.4

The concentration of plasma thiamine was slightly lower than in the preceding experiment. During the infusion of thiamine, the values increased to 14.69 μg/100 ml, later, however, decreased to 11.67 μg/100 ml. A further decrease was observed during the glucose periods. The urinary excretion of thiamine was significantly lower than in the preceding experiment, but higher than in Experiment 1. During thiamine infusion the urinary excretion increased to 38.86 μg/minute, later decreased to 30.50 μg, and showed further decrease during the glucose infusion despite continuous infusion of 20 μg of thiamine hydrochloride per minute.

Table IV. *Experiment 4. Dog Ammy. No thiamine was given before the experiment. Priming infusion of 20 grams of glucose was administered before period 3, sustaining infusion of 1200 mg/minute given during periods 3 and 4.*

	Control periods		Glucose periods	
	1	2	3	4
Plasma thiamine concentration (μg/100 ml)	0.50	0.34	0.45	0.48
Thiamine excretion (μg/minute)	0.22	0.14	0.21	0.20
Thiamine "clearance" (ml/minute)	45	42	44	42
Creatinine clearance (ml/minute)	45	38	41	38
Blood glucose (mg/100 ml)			480	540
Glucose excreted (mg/minute)			169	162

The concentration of plasma thiamine remained low throughout the experiment. The urinary excretion of thiamine remained unchanged during the control periods as well as during the glucose periods.

Table V. *Experiment 5. Dog Ammy. Fifty mg of thiamine hydrochloride were given daily for four days before the experiment. Priming infusion of 20 grams of glucose was given before period 3; sustaining infusion of 800 mg of glucose per minute was given during periods 3 and 4.*

	Control periods		Glucose periods	
	1	2	3	4
Plasma thiamine concentration (μg/100 ml)	0.57			
Thiamine excreted (μg/minute)	0.82	0.84	0.76	0.86
Thiamine «clearance» (ml/minute)	143	146	134	150
Creatinine clearance (ml/minute)	43	38	41	43
Blood glucose (mg/100 ml)			400	310
Glucose excreted (mg/minute)			54	6.1

The concentration of plasma thiamine was slightly higher than in the preceding experiment. The thiamine excretion was significantly higher, but remained unchanged during the infusion and excretion of glucose.

DISCUSSION

Thiamine occurs in the urine only as free thiamine. In blood and tissues thiamine is present mostly as phosphates whereas the concentration of free thiamine is low. The concentration of "total" thiamine (free + phosphorylated) in human blood is about 5 μg/100 ml. The concentration of free thiamine is much lower being usually less than 1 μg/100 ml. Also within the organs thiamine occurs mainly as phosphates. The concentration depends upon the tissue saturation with thiamine. In Hjarde's (1950) study the following concentrations were found in the rat: liver about 8 μg/gram of wet tissue, kidney 6 μg/gram, heart 8 μg/gram, most of the thiamine being present as phosphates. Foa, Weinstein, Smith & Greenberg (1952) found about 10 μg/gram of liver tissue, about 80 per cent being thiamine phosphates. In the author's study (Haugen 1961) 10—13 μg/gram of wet liver tissue was found whereas the kidney concentration was about 8—11 μg/gram. In both these organs the concentration of free thiamine was low, 0.1—0.3 μg/gram (fasted rats).

When administered orally, thiamine is rapidly absorbed from the gut, but even large doses produce only slight and temporary increment of the thiamine concentration of the blood because thiamine is rapidly excreted by the kidneys, phosphorylated, or transformed into non-thiamine compounds. Upon parenteral administration, the concentration of free thiamine and thiamine phosphates increases rapidly. Foa, Smith & Weinstein (1947), after intravenous infusion of 1 mg of thiamine hydrochloride per kg of body weight into dogs, demonstrated an immediate increase of free thiamine to 272 μg/100 ml of blood and an increase of thiamine phospha concentration to 55 μg/100 ml. Both valu declined towards the initial values during t 15 minute experimental period. After intr peritoneal injection of 2 mg of thiamine dai for 14 days to rats, Hjarde (1950), 15 m nutes after the last injection, observed hig values of free thiamine in the liver (16.3 μ gram) whereas the concentration of pho phorylated thiamine was about 8 μg/gra Twenty-four hours after the last injecti the concentration of free thiamine was lo in both organs while the concentration thiamine phosphates was unchanged, indica ing that a rapid excretion or destruction free thiamine had taken place.

Another question that should be tak into consideration is the cell/plasma distrib tion of free thiamine. If thiamine in the blo cells is extracted and excreted during t passage of the blood through the tubul capillaries, it would contribute to a possib tubular excretion. Preliminary studies sho ed that thiamine does not to any significa degree penetrate the red cell membra without becoming phosphorylated; cell tra port, is, therefore, probably of no cons quence. A dephosphorylation of thiami phosphates in the blood cells may, ho ever, occur during the passage of the blo through the tubular capillaries, and th participate in a possible tubular excretion thiamine.

A cautious interpretation of the results therefore, necessary, particularly regardi the computation and evaluation of thiami excretion as a "clearance" of this substan However, some conclusions may be dra

In dog Alma the subcutaneous administ tion of 100 mg of thiamine hydrochlor

y raised the thiamine plasma level from —0.4 μg/100 ml to 0.92 μg/100 ml. After lays, when no parenteral thiamine was en, the concentration of plasma thiamine reased to 0.79 μg/100 ml. The "clearance" hiamine in Experiment 1 was very low, ut 6 ml/minute, the simultaneous creatin- clearance about 45—50 ml. After sub- aneous administration of 100 mg of thia- e hydrochloride daily for four days, the mine "clearance" reached the level of atinine clearance, but after 8 days without itional thiamine, the "clearance" again significantly lower than the endogenous atinine clearance.

The excretion of thiamine in this dog in- ates reabsorption of thiamine in the ules, a reabsorption amounting to 86—88 cent of the amount filtered in the glo- uli. This is in concordance with the find- of low thiamine excretion in humans. a regular diet the excretion of thiamine humans is 100—300 μg/24 hours. A lthy individual (endogenous creatinine rance 110 ml/minute) excreted during a longed period about 10 μg per 100 ml of ne on a diuresis of 1,000 to 1,200 ml. As plasma thiamine values were around 0.6 100 ml, the thiamine "clearance" may be ulated to 12—14 ml/minute. On another asion the excretion of thiamine was 0.15 minute and plasma thiamine 0.22 μg/100 giving a "clearance" of 68 ml/minute. ce the "clearance" of thiamine on both asions was lower than the endogenous atinine clearance, a tubular reabsorption hiamine may be assumed.

A somewhat different pattern was obtained log Ammy. Before parenteral administra- the thiamine "clearance" was equal to the glomerular filtration rate. After sub- cutaneous administration of 50 mg of thia- mine hydrochloride for four days, plasma thiamine increased slightly, and the "clearan- ce" of thiamine increased to values well above the glomerular filtration rate. The higher thiamine "clearance" in this dog may be due to a higher thiamine content of the diet although proof for this supposition is lacking.

After intravenous administration of thia- mine, the concentration of venous plasma thiamine increased rapidly. In dog Alma a priming dose of 2 mg followed by a sustain- ing dose increased the thiamine concentra- tion to 6.71 μg/100 ml, and this value was maintained during the infusion of 10 μg of thiamine hydrochloride per minute. The urinary excretion of thiamine increased from the level of the glomerular filtration rate to about 100 ml/minute. In Experiment 3 the same pattern was obtained, although on a higher level because of the higher dose of thiamine hydrochloride administered. The thiamine concentration increased to 14.69 μg/100 ml of plasma, but later decreased in spite of continuous infusion of 20 μg per minute. Because venous blood was used and the concentration of plasma thiamine was falling during Experiment 3, conclusions should be drawn cautiously, but point in the same direction as in Experiment 2: since the "clearance" of thiamine exceeded the endo- genous creatinine clearance, thiamine was excreted also by the tubules. This tubular excretion of thiamine was, at the higher plasma level in Experiment 3, about 80 per cent of the excreted quantity, at the some- what lower plasma thiamine level in Experi- ment 2 about 60 per cent.

The present study indicates that thiamine is a substance that at low blood concentrations is filtered through the glomeruli and from the tubular lumen brought back to the blood, either by passive diffusion or by active reabsorption. Since thiamine is essential to the organism, active reabsorption is conceivable, a process aimed at preservation of thiamine. That such a view is correct is supported by the finding of very low excretion when thiamine is withheld from the diet. Low excretory values during the administration of moderate or low doses of thiamine may, however, be explained in another way, namely by assuming that thiamine is filtered at the glomerulus, reabsorbed more or less completely in the tubules, and also secreted into the urine by the tubular cells. Thus the mechanism for thiamine excretion at low and at high plasma levels would be identical: at low plasma levels the secretion is relatively slight, at high levels and when administered in excess, the tubular secretion is preponderant. Support for this assumption is provided by the finding of reduced excretion of thiamine after administration of probenecid (Haugen unpublished).

Although the "three-component system" as described by Barclay, Cooke & Kenney (1947) in Homer Smith's (1951) opinion is not proven, several observations point to the presence of such a system, *e. g.* for carinamide (Beyer, Russo, Tillson, Gass & Schuchardt 1949), for urate (Gutman, Yü & Berger, 1959) and for thiosulphate (Bucht 1949).

The third object of this study was the examination of thiamine excretion during glycosuria. When low amounts of thiamine were excreted, as in Experiments 1, 4 and 5, a simultaneous glycosuria did not influe the excretion of thiamine that maintai identical levels both before and after gluc infusion. After infusion of large doses thiamine, the simultaneous glucose infus and excretion did not reduce the thiam excretion in Experiment 2. In Experimen a significant decrease of thiamine excret was observed during the glucose perio This was, however, probably due to falling thiamine plasma levels, not to glycosuria. In this experiment glycosu was moderate, but sufficient amounts glucose were probably reabsorbed for satu tion of the tubules. The most important fi ing, however, was that glucose infusion a excretion did not *increase* the excretion thiamine, either at low or at high plas levels of thiamine. It may be concluded t glycosuria *per se* does not influence the cretion of thiamine, and no deficiency of th mine may be due to glycosuria. This find in the dog agrees well with the results tained in human diabetics (Haugen 196

SUMMARY

A study of the renal excretion of thiam in the dog indicated that thiamine is filte through the glomeruli, reabsorbed in tubules and also secreted by the tubular c both at low and at high plasma levels thiamine. Intravenous administration of cose and subsequent glycosuria did not fluence the excretion of thiamine, either low or at high thiamine excretory levels.

ADDENDUM

Since this paper was accepted for publicatio paper concerning some of the same problems peared (Renal excretion of thiamin by the do

Malnic, A. C. da Silva, R. C. de Angelis & J. Gomes *in* Am. J. Physiol. 198, 1274, 1960). se authors demonstrated thiamine: creatinine rance ratios above unity after intravenous ad- istration of thiamine and also obtained evidence tubular reabsorption of thiamine despite high ma thiamine levels. No study of the renal ex- ion of thiamine at normal plasma levels was ormed.

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FATTY ACIDS OF VERNIX CASEOSA

By E. HAAHTI, T. NIKKARI, A.-M. SALMI and A.-L. LAAKSONEN

From the Department of Medical Chemistry, the Childrens Clinic and the Womens Clinic, University of Turku, Finland

(Received for publication June 24, 1960)

Vernix caseosa is a product of sebaceous glands and/or the amnion (Lajos, Jobst & Bascó, 1950). The protecting and nutritive properties of the vernix have been a subject of investigation as early as 1909 (v. Zumbusch). More detailed results are now available from several studies (Lajos & Szontagh 1948; Wheatley 1954), in which samples are usually fractionated with different extraction procedures. In connection with our earlier studies on the fatty acid composition of earwax (Haahti, Nikkari & Koskinen, 1960) and on the general physiologic properties of *vernix caseosa* on the other hand, it was found of interest to obtain some knowledge of the fatty acid composition of *vernix caseosa.*

METHODS

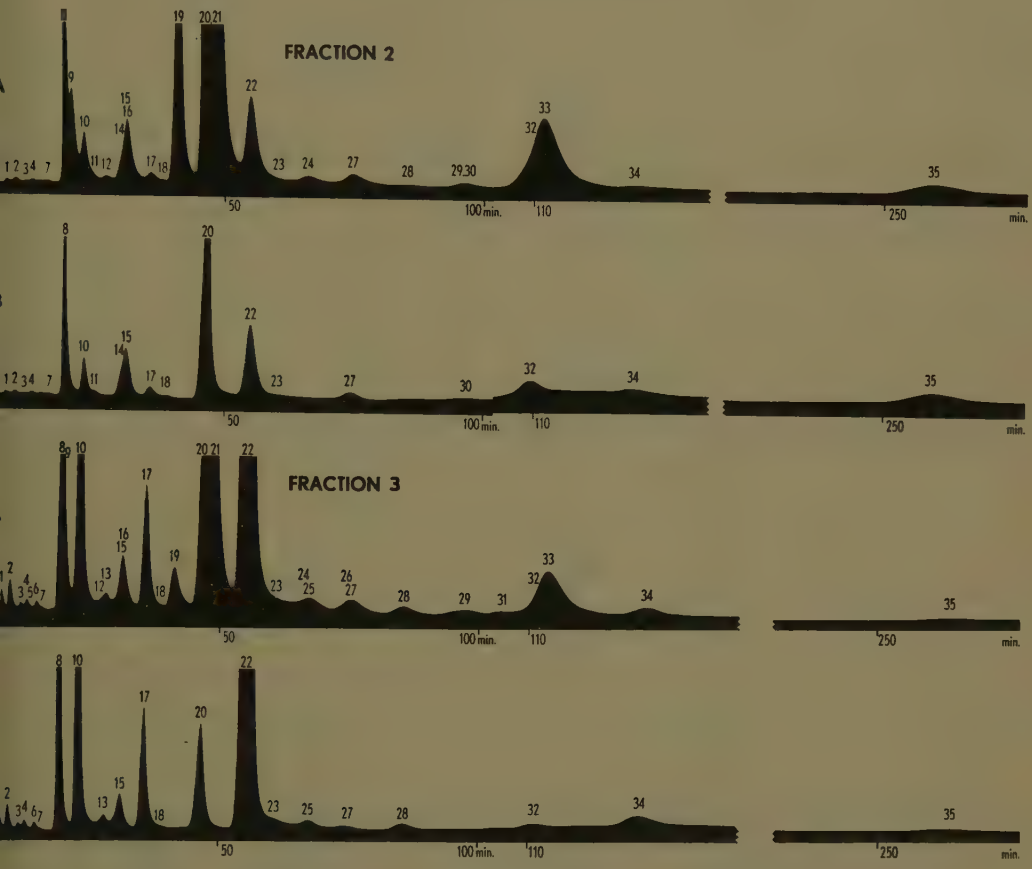
The total lipids of *vernix caseosa* were extrac with 2:1 chloroform-methanol mixture (v/v) purified according to the method of Folch, Asc Lees, Meath & Le Baron (1951). The solvent evaporated in nitrogen flow at 40° C. The lip were weighed, dissolved in light petroleum (30—40° C), and subjected to silicic acid chroma graphy. The silicic acid (Mallinckrodt Chemi Works, 100 mesh) was prepared and stabili according to Sweeley & Moscatelli (1959). solvent mixtures used and the weights of the fr tions obtained from 100 mg of total purified li are shown in Table I.

In fraction 3, the amounts of cholesterol glycerides were determined, and were 40 mg 78 µM respectively.

The fatty acids of aliquots of fractions 2 an were converted to their methyl esters by heat in methanol ("superdry")-HCl (0.7 M) in sea

Table I. *Silicic acid chromatography of vernix caseosa lipids.*

Fraction	Solvent mixture	Name of fraction	Weight mg
1	Light petroleum (b. p. 30—40° C)	Nonpolar fraction	30
2	1 % ethyl ether in light petroleum	Sterol esters	14
3	Chloroform	Free cholesterol Free fatty acids Glycerides	54
4	Methanol	Phospholipids	traces



. 1. Fatty acids of lipid fractions of *vernix caseosa*. Sample 0.1 µl of methyl esters; stationary phase Apiezon L; temp. 190° C; argon pressure 0.6 atm.

A. before bromination, B. after bromination.

ntification of peaks: (1) branched chain saturated C_{12} acid (iso-); (2) *n*-dodecanoic; (3) branch- chain saturated C_{13} acid; (4) branched chain saturated C_{13} acid (anteiso-); (5) mono-unsaturated acid; (6) *n*-tridecanoic; (7) highly branched saturated C_{14} acid; (8) branched chain saturated acid (iso-); (9) mono-unsaturated C_{14} acid; (10) *n*-tetradecanoic; (11) highly branched satur- d C_{15} acid; (12) diunsaturated C_{15} acid; (13) branched chain saturated C_{15} acid; (14) branched in saturated C_{15} acid (iso-); (15) branched chain saturated C_{15} acid (anteiso-); (16) mono- aturated C_{15} acid; (17) n-pentadecanoic; (18) highly branched saturated C_{16} acid; (19) diun- ırated C_{16} acid; (20) branched chain saturated C_{16} acid (iso-); (21) mono-unsaturated C_{16} acid;) *n*-hexadecanoic; (23) highly branched saturated C_{17} acid; (24) diunsaturated C_{17} acid; (25) nched chain saturated C_{17} acid; (26) mono-unsaturated C_{17} acid; (27) branched chain saturat- C_{17} acid (anteiso-); (28) n-heptadecanoic; (29) triunsaturated C_{18} acid; (30) highly branched ırated C_{18} acid; (31) diunsaturated C_{18} acid; (32) branched chain saturated C_{18} acid (iso-); (33) no-unsaturated C_{18} acid; (34) *n*-octadecanoic; (35) branched chain saturated C_{20} acid (iso-).

tubes at 67° C for two hours. The esters were then extracted with light petroleum, and after evaporation of the solvent, sublimated *in vacuo* at 80° C.

Samples of fatty acid methyl ester mixtures of fractions 2 and 3 were analysed in a gas-chromatography apparatus constructed in this laboratory according to James (1958). A 120 cm long glass column of 4 mm diameter with a stationary pha of 10 per cent Apiezon L (w/w) in Celite 545 (1 —120 mesh) was used, together with an argon io ization detector (Haahti, Nikkari & Kulonen 196 The column and the detector were maintained a temperature of 190° C. The chromatograms a shown in Fig. 1.

Table II. *Fatty acid composition of vernix caseosa.*

Peak No	Log. ret. time	Ret. time relative to methyl myristate	"carbon number"	Number of C-atoms
		Saturated acids:		
2	0.986	0.422	12	12
6	1.171	0.643	13	13
10	1.362	1.00	14	14
17	1.553	1.55	15	15
22	1.739	2.39	16	16
28	1.930	3.70	17	17
34	2.119	5.72	18	18
		Unsaturated acids:		
5	1.124	0.573	12.75	13 (mono-)
9	1.312	0.891	13.74	14 (»)
12	1.435	1.18	14.38	15 (di-)
16	1.496	1.36	14.71	15 (mono-)
19	1.615	1.79	15.35	16 (di-)
21	1.681	2.09	15.74	16 (mono-)
24	1.820	2.87	16.43	17 (di-)
26	1.869	3.22	16.69	17 (mono-)
29	1.981	4.16	17.28	18 (tri-)
31	2.017	4.52	17.46	18 (di-)
33	2.055	4.93	17.67	18 (mono-)
		Branched chain saturated acids:		
1	0.906	0.351	11.59	12 (iso-)
3	1.072	0.513	12.47	13
4	1.114	0.565	12.69	13 (anteiso-)
7	1.202	0.691	13.16	14 (highly)
8	1.283	0.834	13.59	14 (iso-)
11	1.396	1.08	14.18	15 (highly)
13	1.447	1.22	14.46	15
14	1.473	1.29	14.59	15 (iso-)
15	1.493	1.35	14.69	15 (anteiso-)
18	1.590	1.69	15.21	16 (highly)
20	1.664	2.02	15.60	16 (iso-)
23	1.784	2.64	16.23	17 (highly)
25	1.826	2.91	16.46	17
27	1.871	3.22	16.70	17 (anteiso-)
30	1.982	4.17	17.28	18 (highly)
32	2.042	4.78	17.60	18 (iso-)
35	2.418	11.4	19.60	20 (iso-)

ne recorded peaks were identified as follows:
Comparing the retention times with those of
rn substances.
Eliminating the unsaturated esters by brom-
on.
Expressing the retention times as relative to
yl myristate and comparing with known re-
e retention times.
Plotting the logarithms of retention times
ist number of carbon atoms, thus making use
e fact that straight lines are formed by homo-
es and the "carbon numbers" (Woodford &
Gent 1959) can be obtained.
he logarithms of the observed retention times,
relative retention times to methyl myristate,
hé "carbon numbers" are presented in Table II.
ne case of the branched chain acids, the given
es indicate the presence of 4 types of branching.
exact identification of these compounds, how-
has not been practicable with present meth-
It might be possible that some of these peaks
caused by higher free alcohols.

RESULTS

he results from the fractionation of the
ix caseosa lipid are in agreement with
ier data (Wheatley 1954; Lajos &
ntagh 1948). The fatty acid composition
ost coincides with the corresponding
lts from sebum (James & Wheatley
6) and cerumen (Haahti *et al.* 1960).
vever, the total number of different acids
ernix caseosa is conspicuous as compared
1 the former.

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CYSTINURIA IN SWEDEN V.

INFLUENCE OF TEMPERATURE ON THE SEPARATION OF BASIC AMINO ACIDS BY HIGH-VOLTAGE PAPER ELECTROPHORESIS[1]

By L. HAMBRÆUS

From the Department of Metabolic Research, Wenner-Gren Institute of Experimental Biology, University of Stockholm, Stockholm, Sweden

(Received for publication June 9, 1960)

Separation of the basic amino acids has been a problem for many years, since conventional paper chromatographic methods do not, as a rule, give satisfactory results. Consequently, a great advance was made when Harris & Warren (1954) succeeded in separating them by ionophoresis in filter paper. They used a buffer of pH 11.5 and a potential difference of 185 V for 17 hours.

The introduction of high-voltage electrophoresis (Michl, 1951) provided a more rapid method for separation of amino acids, and diminished the disturbing effect of diffusion. This one-way separation has subsequently been refined and used in apparatus of various types (Kichhöfen & Westphal 1952 a; Heilmeyer, Clotten, Sand, Sturm & Lipp 1954; Werner & Westphal 1955; Gross 1955, 1956; Wieland & Pfleiderer 1955, 1957; Nöller, Stelgens & Kieser 1957; Visakorpi & Puranen 1958: Atfield & Morris 1960). A large number of amino acids have, however, been difficult to separate by these methods.

Separation has usually been done at a fa ly low pH, using long strips of paper separate the basic amino acids (Visakorpi Puranen 1958). Even with these techniqu the separation of ornithine and lysine h been difficult. Several workers have ther fore tried techniques with a combination high-voltage electrophoresis and pap chromatography (Kickhöfen & Westph 1952 b; Honegger 1956; Dixon, Kauffm & Neurath 1958; Efron 1959).

In connection with a study on cystinur I was interested in separating the ba amino acids arginine, lysine and ornithi by high-voltage electrophoresis at a high pH (pH 9.7). I then observed that consid able spreading of the spots occurred when temperature of +5° C was applied. If, ho ever, the temperature was raised to +35 40° C hardly any spreading took place, m gration increased, and good separation w obtained in 1 hour.

As described in the following, this tec nique has proved useful in the differen diagnosis of homozygous and heterozygo cystinuria.

[1] The investigation was aided by grants from Karolinska Institutets reservationsanslag and by a grant to Dr. H. Boström from the Swedish Medical Research Council.

METHODS

Electrophoresis was carried out in an apparatus[1] re the paper strip lies horizontally, and is press- between two glass surfaces on a cooling block luminum, in which water circulates.

Paper electrophoresis was performed on What- No. 1 filter paper (21.5 × 50 cm) in a 0.2 *M* um borate buffer, adjusted to pH 9.7 with um hydroxide. The amino acid solutions[2] and urine samples were applied with micropipettes line in the middle of the paper, the amount of e applied corresponding to 10 µg of creatinine. The paper was moistened with the buffer and d between blotting paper, after which electro- esis was performed at a constant potential of V/cm for 1 hour at the desired temperature. temperature was controlled by circulation of er from a thermostatic bath with a Thermomix[3] he cooling plate of the apparatus.

After drying the papers in a stream of hot air n an electric fan, the chromatograms were deve- d according to Harris, Mittwoch, Robson & ren (1955 a) by dipping them rapidly in a solu- consisting of 0.4 g of ninhydrin, 95 ml of one, 5 ml of glacial acetic acid and 5 ml of er. The paper was dried in a stream of hot air was then heated in an oven at 110° C for 3—5 ites.

The urine samples had been sent by post from ons in the country; they were collected in 50 ml les, containing a crystal of thymol to prevent growth of microorganisms.

RESULTS

The results show that raising the tem- ature from +5° C to +40° C increased possibility of separating the basic amino ls arginine, lysine and ornithine by high- age electrophoresis at pH 9.7. As seen

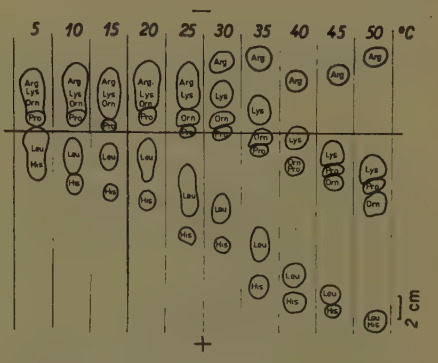

Fig. 1. *Diagram of the electrophoretic behaviour of six amino acids at different temperatures.*

(0.2 *M* sodium borate buffer, pH 9.7, 30 V/cm, 1 hour.) The amino acids were applied on the line[4]. The numerals denote the temperature of the water circulating in the cooling plate of the apparatus. The diagram represents the collective results of a series of electropherograms.

in Fig. 1, a rise in temperature not only produced more rapid migration, but also caused displacement of the migration of the amino acids in relation to the starting point. At +5° C, ornithine, lysine and proline migrated to the cathode, but at +40° C ornithine and proline migrated to the anode, whereas lysine remained on the application point.

When electrophoresis was carried out at 30 V/cm and pH 9.7 for 1 hour at +35—40°C, no difficulties were encountered in separating arginine, lysine and ornithine. The following amino acids moved more rapidly to the anode than ornithine: β-aminoisobutyric acid, γ-aminobutyric acid, β-alanine, citrulline, his-

[1] Constructed by Dr. Igor Cedergren, Svenska Forskningsinstitutet.

[2] Set no. 2056, Shandon Scientific Co. Ltd., London.

[3] B. Braun, Melsungen, West Germany.

[4] The abbreviations in Figs. 1 and 2 are the same as those used by Brand & Edsall (1947). Arg = arginine; His = histidine; Leu = leucine; Lys = lysine; Orn = ornithine; and Pro = proline.

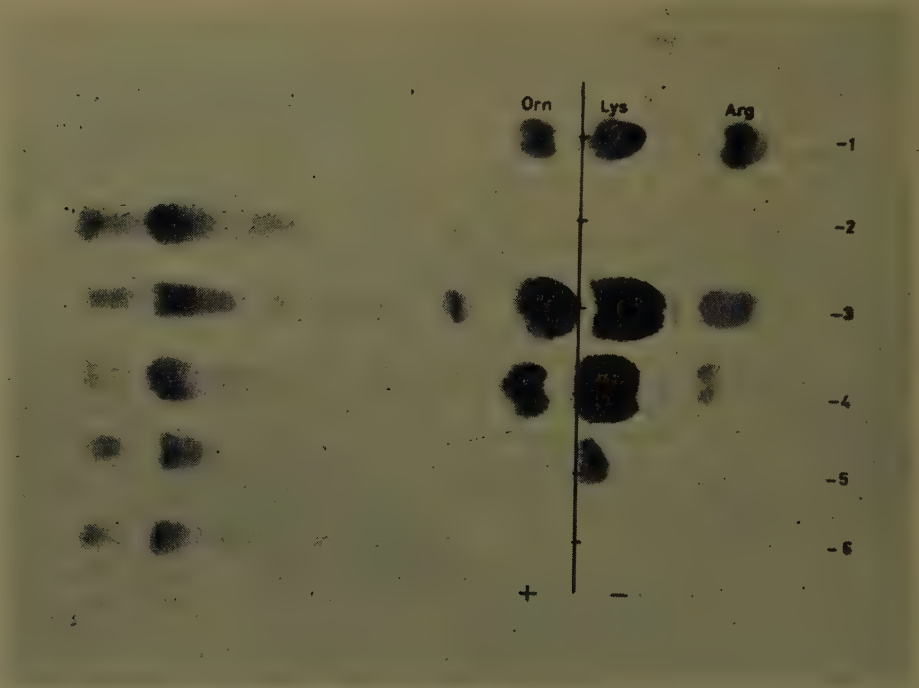

Fig. 2. *Electropherogram of urine samples.*

Paper electrophoresis (0.2 *M* sodium borate buffer, pH 9.7, 30 V/cm, 1 hour, +40° C) on urine specimens from a healthy adult male (2), an adult male with classical cystinuria (3), a 9-year-old girl (4) and her mother (5), both of whom had a positive nitroprusside reaction, and the father of the girl (6), who had a negative nitroprusside reaction. Standa solutions of arginine, lysine and ornithine are cluded as a comparison (1). The amount of ur applied corresponds to 10 µg of creatinine, exc No. 4, which corresponds to 5 µg.

tidine, methyl histidine, leucine and isoleucine. Proline, however, migrated at about the same rate as ornithine, as seen in Fig. 1.

Fig. 2 shows the electrophoretic urinary pattern in a family in which the daughter (4) and mother (5) had a positive nitroprusside reaction, whereas the father (6) had a negative one. As a comparison, the pattern in a healthy adult male (2) is shown, as well as that in an adult male with classical cystinuria (3), characterized by cystine calculus formation and urinary excretion of cysti lysine, arginine and ornithine.

It is then seen that the daughter has pattern of classical cystinuria, whereas mother has an accentuated lysine spot or According to Harris, Mittwoch, Robson Warren (1955 b), the daughter can be garded as a homozygote of cystinuria and mother as a heterozygote. The pattern of father's urine is, however, similar to t obtained in healthy subjects.

DISCUSSION

Judging by the results, a temperature of 35—40° C is most convenient for obtaining good separation of the basic amino acids der these conditions. The only amino acid ich is difficult to separate from ornithine proline. This is a minor drawback, since oline gives a yellow colour with ninhydrin ich can easily be distinguished from the rple colour given by ornithine. Leucine and minobutyric acid, which, according to lier reports, have been difficult to separe from ornithine (Harris, Mittwoch, Rob- & Warren, 1955 a) move more rapidly to anode than ornithine.

As little as 1 μg of the basic amino acids easily be detected after electrophoresis been carried out.

Quantitative estimation of the colour deved in the ninhydrin reaction, after elution 50 per cent ethanol, showed no signs of increased loss in electrophoresis at a gher temperature, compared to that at a ver temperature.

The method seems to be an improvement those now available for the diagnosis of tinuria, since it allows rapid differentian between the cystine-lysine-arginine-ithine pattern and the cystine-lysine ttern of the urine of cystinurics.

SUMMARY

The influence of temperature on the aration of basic amino acids by high-tage electrophoresis at pH 9.7 has been died. Good separation of arginine, lysine l ornithine is obtained by paper electroresis at 30 V/cm and pH 9.7 at +40° C for 1 hour. The method has been used to differentiate between homozygous and heterozygous cystinuria.

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IN VITRO EFFECTS OF HEPARIN AND CHONDROITIN SULFURIC ACID B ON THE GENERATION OF THROMBIN

By B. J. GROSSMAN[1] and T. ASTRUP

From the Department of Pediatrics, University of Chicago, Illinois, and the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark

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Marbet & Winsterstein (1951, 1952) olated from lung a mucopolysaccharide th anticoagulant properties which was med β-heparin. Meyer & Rapport (1951) oposed the name chondroitin sulfuric acid (CSA-B) for this compound. This poly-ccharide has been isolated from a variety sources including lung, Marbet & Winter-in (1951, 1952), gastric mucosa, Smith & llop (1953), and skin, Meyer & Chaffee 941), Schiller, Mathews, Jefferson, Ludo-eg & Dorfman (1954). The anticoagulant operty of CSA-B was demonstrated by arbet & Winterstein (l. c.) and Studer & interstein (1951) using a thrombin method assay. Yamashina (1954) found CSA-B be an active anticoagulant only with the rombin method of Studer & Winterstein 951). In a more detailed investigation, ossman & Dorfman (1957) found that at v thrombin concentration CSA-B had a rked antithrombin activity when compared with heparin. When higher thrombin concentrations were used CSA-B was less active than heparin and appeared to inhibit the natural antithrombic properties of normal plasma. It was also noted that CSA-B is an anticoagulant in human whole blood if used in sufficient concentrations.

Heparin has been known to inhibit thrombin generation at concentration of 0.5 to 1.0 unit per ml of plasma, Biggs, Douglas & Macfarlane (1953), MacMillan & Brown (1954), Jorpes (1955). The purpose of this investigation is to compare the effects of CSA-B and heparin on the generation of thrombin.

METHODS

The method of Sjölin (1956) was chosen for measurement of thrombin generation. The technique was followed strictly except that the tubes used for the test were of an internal diameter of 15 mm and a series of 20 tubes was used. Normal citrated human plasma was used in all experiments.

One ml of the plasma sample and 1 ml of normal saline were mixed and allowed to reach the temperature of the water bath, 37° C. One ml of 0.025 *M* $CaCl_2$ was added to this tube and aliquots of 0.1 ml were removed each minute and reacted

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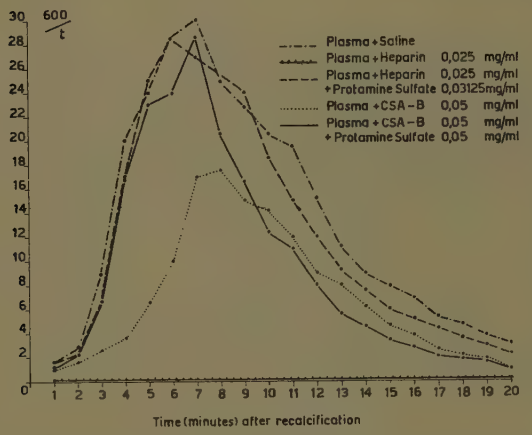
600/t
Plasma + Saline
Plasma + Heparin 0,025 mg/ml
Plasma + Heparin 0,025 mg/ml
+ Protamine Sulfate 0,03125mg/ml
Plasma + CSA-B 0,05 mg/ml
Plasma + CSA-B 0,05 mg/ml
+ Protamine Sulfate 0,05 mg/ml
Time (minutes) after recalcification

Fig. 1. Curves representing thrombin generation demonstrating effects of heparin, CSA-B and mixtures of these compounds with protamine sulfate on generation of thrombin. (For details see text.)

with 0.4 ml of a 0.15 per cent fibrinogen solution. The clotting time (t) was noted.

A curve relating the clotting time of the fibrinogen solution (t) and the incubation time after recalcification was plotted (Fig. 1). An arbitrary unit of thrombin was chosen for the purpose of comparing one experiment with another. This unit was taken as the reciprocal value of the clotting time of the fibrinogen solution (t) measured in seconds and calculated as 600/t. The concentration of thrombin is nearly inversely proportional to the clotting time of the fibrinogen solution, Astrup & Darling (1942). The curve for normal plasma (Fig 1) shows that the thrombin formation is slow during the first two minutes after recalcification. After this initial slow period the thrombin concentration increases rapidly and reaches a maximum 5 to 7 minutes after recalcification. Thereafter the thrombin is inactivated and at the end of 20 minutes is practically all destroyed. The substances to be tested were dissolved in saline to the desired concentration and added to the plasma in the saline diluent. The following compounds were tested: 1) Heparin — 132 IU/mg, supplied by Abbott Laboratories; 2) CSA-B, supplied by Hoffman—La Roche through the courtesy of Dr. A. Winterstein.

RESULTS

When CSA-B or heparin was added to t citrated plasma and the generation of thro bin measured, the effects on thrombin ge ration were found to vary according to t concentration of polysaccharide in the rea tion mixture. In Table I the effects of hep rin and CSA-B at various concentrations a compared. Heparin at a concentration 0.001 mg per ml has a significant delayi effect on thrombin generation, it suppress the amount of thrombin generated and i creases the recalcification time (RCT). Co parable effects of CSA-B are not evident u til a concentration of 0.05 mg per ml w reached. When 0.005 mg per ml of CSA was added to plasma no demonstrable var tion of the curve from normal was observe At increasing concentrations of CSA-B (0.0 0.05, 0.1 and 1.0 mg per ml) it can be sho that the first effect was a diminution in t amount of thrombin generated and wi higher concentration of CSA-B an creasing time for the generation of thromb until at 1.0 mg of CSA-B per ml of plas very little thrombin is generated.

When the same experiment was perfor ed using heparin, Table I shows that 0.0005 mg there was no effect on the gene tion of thrombin. However, at 0.001 there was a marked delay in thrombin ge ration and at 0.005 mg no thrombin w generated. Table I also shows the effect various concentrations of heparin and CS B on the recalcification time (RCT).

A series of experiments was performed test the effect of protamine sulfate on generation of thrombin. Protamine sulfate known to inhibit the anticoagulant p perties of heparin, Fischer (1935), Ch

f & Olson (1937). It was found that at 5 mg of protamine sulfate per ml there s no effect on the thrombin generation ve. At 0.1 mg per ml there was a short ay in thrombin generation but there was decrease in the height of the curve (a asure of the amount of thrombin gene- ed). At 0.5 mg per ml of protamine sul- e there was considerable delay in thrombin neration, but once begun, the final height the curve was undiminished.

A series of experiments was performed to amine the effect of mixing protamine sul- e with CSA-B and heparin and testing e effects of the mixtures on the generation thrombin.

Fig. 1 illustrates the results of such an periment. It can be seen that at a ratio of 1.25 of heparin to protamine sulfate the effect of heparin on thrombin generation can be abolished. At a ratio of 1:1, CSA-B to protamine sulfate, the inhibiting effect of CSA-B on thrombin generation disappeared.

A final series of experiments was performed to show that heparin and CSA-B added to the plasma do not merely inactivate the thrombin immediately on being formed but actually inhibit thrombin generation. A sufficient quantity of CSA-B and heparin were incubated with the plasma so that no thrombin was generated after recalcification. At the end of five minutes of incubation, protamine sulfate was added and the reaction mixture tested for the generation of thrombin. In both instances, if sufficient protamine sulfate was added, the generation of thrombin proceeded normally. This experiment would indicate that the generation of throm-

Table I. *Thrombin generation at various concentrations of polysaccharide.*

ncentration of lysaccharide mg per ml.	CSA—B		Heparin	
	Description of thrombin generation curve	RCT* increase over normal	Description of thrombin generation curve	RCT* increase over normal
.0005	Normal	0	Normal	25
.0010	»	0	Delayed generation, 9 min. Depressed total generation.	25
.0050	No delay in generation. Slightly depressed curve.	0	Delayed generation, 11 min. Very depressed generation.	845
.0100	»	10	No generation of thrombin	1800
.0500	Delayed generation. Moderate depression of curve.	207	»	Incoag.
.1000	Delayed generation, 3 min. Moderate depression of curve.	125	»	»
.5000	Delayed generation, 9 min. Marked depression of curve.	450	»	»
1.0000	Delayed generation, 19 min. Very marked depression	1310	»	»

* RCT = recalcification time.

bin is merely inhibited by heparin and CSA-B and that thrombin is not destroyed, and when the inhibitor is released by addition of protamine sulfate the reaction proceeds normally.

The amount of protamine sulfate necessary to neutralize completely the effect of heparin on thrombin generation was less than that which would itself interfere with thrombin generation. With CSA-B, when the experiment was performed at a concentration of 1.0 mg per ml, only 0.10 mg per ml of protamine sulfate could be added. More protamine sulfate would itself have an inhibiting effect. Generation of thrombin began promptly even though the curve did not rise to the same height as the normal. This indicates that complete neutralization of CSA-B activity was not achieved at this ratio of protamine sulfate to CSA-B.

DISCUSSION

The formation of thrombin can be followed directly by appropriate methods, Macfarlane & Biggs (1953), Pitney & Dacie (1953), instead of indirectly by disappearance of prothrombin. The thrombin generation test of Sjölin (1956) was chosen because of ease of performance and the fact that so little handling of the blood is required.

Biggs *et. al.* (1953) have studied the effects of heparin on the thromboplastin generation test. They found that 0.15 to 1 unit of heparin per ml of plasma prevented the intrinsic thromboplastin formation in recalcified human plasma. The same effect was found by MacMillan & Brown (1954). Using the present method of total thrombin generation, these results are confirmed. In addition, the effect of CSA-B is demonstra ed. By the thrombin generation test we ha found that between 0.005 mg per ml a 0.010 mg per ml of heparin inhibits thro bin generation to about the same degree 1.0 mg per ml of CSA-B, (Table I).

Yamashina (1954) denied any an coagulant effect of CSA-B *in vitro* or *in vi* Grossman & Dorfman (1957) reported anticoagulant effect on whole human blo *in vitro* due to CSA-B. This anticoagula effect of CSA-B can be explained in part the basis of the inhibition of generation thrombin. The reversibility of the effects thrombin generation of heparin and CSA by protamine sulfate may indicate that the compounds produce their effects in a co parable manner.

SUMMARY

1) Chondroitin sulfuric acid B (CSA-B and heparin in sufficient concentration i hibit generation of thrombin.

2) Heparin is a more powerful inhibit of thrombin generation than is CSA-B.

3) Protamine sulfate reverses the i hibiting effects of CSA-B and heparin thrombin generation.

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Scandinav. J. Clin. & Lab. Investigation 84—86, 13, 1961.

A NOTE ON THE DETERMINATION OF PHENYLPYRUVIC ACID IN URINE

By P. J. CHRISTENSEN

From the Biochemical Institute, Aarhus University, Denmark

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Penylpyruvic acid in urine is determined by a number of modifications of two fundamentally different methods. The original detection by Følling in 1934 of phenylpyruvic acid in urine based on a color reaction with ferric ions has been applied in various ways to the quantitative estimation of the compound, the most recent of these methods was published by Følling og Sydnes (1958)[1]. Another method, first published by Penrose & Quastel (1937), is less specific, based as it is on the common reaction of ketones with 2,4-dinitrophenylhydrazine. The color developed by this method is, however, rather stable in contrast to the ferric complex color.

In the present paper phenylpyruvic acid is determined as the difference between total urinary dinitrophenylhydrazones and the dinitrophenylhydrazones of the alkali-stable ketones. The method has been compared to the method of Følling & Sydnes for the assay of phenylpyruvic acid in a series of urines from a phenylketonuric person, the former method yielding in average 101 per cent the latter.

METHODS

Determination of phenylpyruvic acid with dinitrophenylhydrazine.

P r i n c i p l e : Photometric determination of t color developed by dissolving a precipitate of ph nylpyruvic acid 2,4-dinitrophenylhydrazine in alk As blank value is used a sample of urine fr which the phenylpyruvic acid is removed by oxi tion with peroxide at alkaline reaction.

P r o c e d u r e : To a test tube is added 200 urine and 500 µl distilled water, followed by 100 of 2 per cent H_2O_2 (for blank value determinatio and then, immediately, 50 µl of 2 N NaOH. In t samples where the phenylpyruvic acid is to be p served, the peroxide is substituted by water. T samples are gently shaken a couple of times. Af 10 minutes, acid is added in excess (100 µl 2 N HCl), and then 1000 µl hydrazine reagent saturated solution of 2,4-dinitrophenylhydrazine 1 N HCl) is added. The sample is left stand for 30 minutes at room temperature with occasio shaking. Then, after the addition of 4 1 N NaOH, the sample is immediately diluted 25 ml with distilled water. With the alkali addit a color is developed which varies from yellow reddish brown with the concentration of phen pyruvic acid. Maximum intensity is attained w

[1] After this paper was submitted for publication a new modification of the Følling method has appeared (Meulemans, 1960).

kly (within 2 minutes, *cf.* Lu, 1939), and there- r the extinction decreases slowly with a linear of 15 per cent during 45 minutes. The color nsity is read in a Beckman spectrophotometer at 440 mμ, generally about 10 minutes after dilution of the sample. In case of delayed read- it is possible to make a time correction. The ples are read against a reagent blank, and all yses are performed in triplicate.

n a pure solution of phenylpyruvic acid a linear tionship between extinction and concentration is d, E = 1.000 corresponding to 206 mg/100 ml. Different amounts of phenylpyruvic acid added normal urine have been recovered by 97 ± 8.3 cent (S.D.).

Determination of phenylpyruvic acid in 6 urine ples from phenylketonurics has been performed a deviation of two subsequent determinations ± 2.9 per cent (S.D.).

The hydrazones of "non-oxidizable compounds" he urine (blank value hydrazones) amount to 5 mg/100 ml, calculated as phenylpyruvic acid, rine from normal and phenylketonuric persons. n two normal urines the amount of "oxidizable erial" is equivalent to 5—6 mg/100 ml of nylpyruvic acid. In a single experiment about thirds of this amount was found to be extract- with ether from urine acidified to pH = 1 with . The major part of the ketone material in the r extract was easily oxidizable, corresponding phenylpyruvic acid content in normal urine of ut 1.5 mg/100 ml. The oxidizable part of the due is only about 0.5 mg/100 ml (calculated henylpyruvic acid).

Determination of phenylpyruvic acid with ferric . The analysis is performed as described by ing & Sydnes (1958). 200 μl samples of urine used. The standard curve is linear as described he authors, but the extinction curve found for tions of phenylpyruvic acid in urine is not, the ar extinction coefficient being depressed at low entrations. It has therefore been necessary to ulate the phenylpyruvic acid concentration in ological urines from a special standard curve ced out through the addition of known amounts henylpyruvic acid to urine. The determination

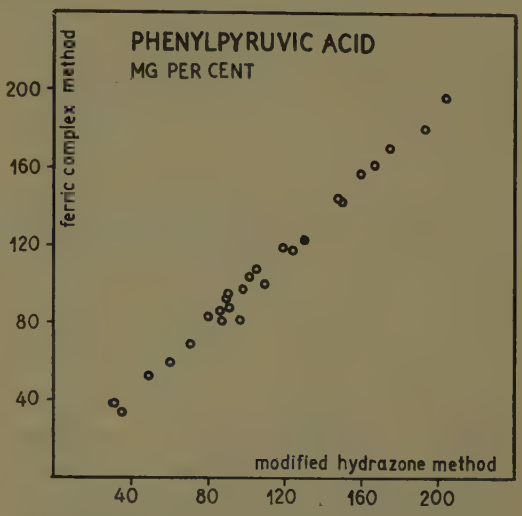

Fig. 1. Comparison of the ferric complex method (Følling & Sydnes method) and the modified phenylpyruvic acid 2,4-dinitrophenylhydrazone method for the determination of phenylpyruvic acid in urine.

of phenylpyruvic acid thus performed shows an accuracy of ± 2.3 per cent (S.D.).

Comparison of the methods. The modified dinitrophenylhydrazone method has been checked by comparison with the method of Følling & Sydnes which is based on an entirely different principle. Fig. 1 shows the correspondance of the two methods when used for the determination of phenylpyruvic acid in a series of urines from a phenylketonuric person.

The two methods are about equally sensitive and reproducible.

DISCUSSION

A considerable number of substances which react with 2.4-dinitrophenylhydrazine are known to occur in small amounts in normal urine. This makes the original method of Penrose & Quastel yield too high values for phenylpyruvic acid, since it in-

cludes all these substances, *cf.* Berry & Woolf (1952). The prevailing ketones of normal urine are α-ketoglutaric and pyruvic acid which occur in amounts of about 2 and 0.2—2 mg per cent, respectively (Friedemann & Haugen 1943; Cavallini, Frontali & Toschi 1949). These two keto-acids, being considerably more stable than phenylpyruvic acid, are included in the blank values obtained by the modified hydrazone method here presented. In some pathological cases, such as diabetes mellitus, starvation and other ketogenic conditions, where the ketones acetoacetic acid and acetone may occur in abnormal amounts in the urine, one cannot safely use any of the hydrazone methods, *cf.* Penrose & Quastel (1937) and Klein (1941).

The advantage of the modified hydrazone method as presented is that it is considerably quicker and less complicated than the ferric complex method, and thus especially fit for routine analysis.

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dinav. J. Clin. & Lab. Investigation 87—99, 13, 1961.

RENAL HANDLING OF POTASSIUM STUDIED BY ORDINARY AND MODIFIED STOP FLOW TECHNIQUE

By K. AUKLAND and F. KIIL

From the Institute for Experimental Medical Research, University of Oslo and Medical Department VII, Ullevål Hospital, Oslo, Norway

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Under normal conditions the renal potassium clearance is less than the filtration rate, indicating tubular reabsorption of potassium. After ingestion of potassium or agents which increase potassium excretion, however, potassium clearance higher than the filtration rate can be produced, showing that the renal tubules are able to secrete potassium (Berliner & Kennedy 1948; Foulks & Gilman 1958). Indirect evidence suggests that most of the filtered potassium is reabsorbed by the tubules, and that a distal secretion of potassium accounts for the main part of the urinary potassium (Berliner, Kennedy & Orloff 1951; Davidson, Levinsky & Berliner 1958). This has, however, only been demonstrated in the presence of high concentrations of poorly reabsorbable anions, which are likely to increase the electric potential over the cell membrane.

Recently the stop flow method of Malvin, Wade & Sullivan (1958 a) has been used to localize tubular potassium secretion and reabsorption. In the ordinary stopflow technique, mannitol is used as a diuretic agent. During 4 to 8 minutes of ureteral occlusion a number of reabsorptive and excretory tubular functions continue as shown by multiple sampling of the urine accumulated in the kidney during the stop-period. The stop flow patterns showed that potassium is reabsorbed in the proximal part of the distal tubule and secreted farther distally, in or slightly distal to the sodium-reabsorbing segment (Malvin *et al.* 1958 a; Pitts, Gurd, Kessler & Hierholzer 1958).

During these experimental conditions the osmolar concentration of the urine samples is only slightly increased above serum level (Malvin & Wilde 1959), suggesting that the procedure in some way interferes with the urineconcentrating mechanism. However, when diuresis is induced by other means than the administration of non-reabsorbable diuretic agents (water, hypertonic sodium chloride, pitressin or mercuhydrin), the osmolar concentration increases considerably in the urine portions accumulated in the collecting ducts (Kiil & Aukland 1960 a, b). By this modification of the stopflow technique it is possible to distinguish between urine portions arrested in the collecting ducts and portions delayed in the distal tubules. Furthermore, since the creatinine concentration index, by this modification of stop flow, reaches values met with during strictly physiological conditions, with ensuing high concentration gradients for other urine

solutes, it was assumed that the distal stop flow pattern for potassium excretion would be altered. Since water abstraction from the tubules is not restricted as in the presence of mannitol, the glomerular filtration continues even when the stop flow is prolonged to twenty minutes (Kiil & Aukland 1960 a). Additional information about proximal tubular events can, accordingly, not be gained. This paper is therefore mainly concerned with comparing the distal excretion patterns for potassium with those obtained by the ordinary stop flow technique. A re-examination of the potassium excretion patterns obtained by ordinary mannitol stop flow technique revealed that the patterns were much more variable than apparent from previous studies.

METHODS AND MATERIAL

Experiments were performed on mongrel dogs, weighing 12—22 kilos, anesthetized with sodium pentobarbital *i. v.*, 25 mg per kilo, and with subsequent small doses for the maintenance of light anesthesia. The experimental procedure followed was mainly as outlined by Malvin *et al.* (1958). Polyvinyl catheters of largest possible bore were inserted through a flank incision into one of the ureters and the tip tied in place at the pelvioureteral junction, so that leakage along the catheter could be avoided.

The experiments were performed during various types of diuresis. Mannitol was the diuretic agent in 29 stop flow series on 15 dogs. In two of these experiments the renal artery and vein were clamped 15 seconds after the occlusion of the ureteral catheter and unclamped 15 seconds before reinstatement of urine flow. 6 stop flow series were performed in 4 dogs during water diuresis, induced by the administration by stomach tube of 500—600 ml water followed by *i. v.* infusion of hypotonic solutions. In 9 experiments in 6 dogs diuresis was induced by Mercuhydrin or large doses of Pitressin (Parke-Davis & Co.) as previously described (Kiil & Aukland 1960 a). In 8 stop flo series in 3 dogs, high rates of urine flow a potassium excretion were induced by the infusi of 3 per cent NaCl at a rate of 8 ml/min and Diamox administered *i. v.* as a priming dose 100 mg followed by a sustaining infusion of 2 mg/min.

The various loads were maintained by continuo *i. v.* infusion at a rate of 2 to 8 ml per minu using electric infusion pumps. Creatinine, and aminohippurate (PAH) in amounts expected insure complete extraction were added to the fusion fluid.

When urine flow had stabilized, the stop flo periods were preceded and followed by two or thr clearance periods, each of two minutes duratio The catheter was clamped for periods varying fro 4 to 10 minutes. In the course of the two minut following release of the clamp about twenty uri samples of approximately equal volume were c lected. New filtrate was indicated by inulin jected *i. v.* 45 seconds before the clamp was releas

Sodium and potassium were analyzed with Baird indirect flame photometer, creatinine by t method of Bonsnes and Taussky (1945), PA according to Smith, Finkelstein, Aliminosa & Gr ber (1945), inulin according to Rolf, Surthshin White (1949), urea according to Conway (193 Urine osmolality was not measured, and the molar concentration of blood and urine was estim ed as the osmolar concentration of urea, creatini and twice the sodium and potassium concentrati measured in mEq/l. By this procedure the osmol concentration of urine is slightly underestimat When comparing the urine osmolarity with t urine osmolar concentration estimated in this w as given in the tables of Levinsky, Davidson Berliner (1959) the difference, however, is small to invalidate any of the conclusions dra in this paper. Osmolar concentrations were estimated in experiments on mannitol diures where they repeatedly have been shown not differ essentially from the osmolar concentration plasma (Kessler, Hierholzer, Gurd & Pitts, 19 Malvin & Wilde, 1959).

Nomenclature: At the bottom of each fig the concentration index for creatinine (U/P_{Cr}) samples of urine obtained in control periods and

e accumulated during stop, indicates the tubular r abstraction. The curve for U/P_{Na} mainly es for reference, since urine portions of low um concentration are assumed to have been ved in the distal tubules (Malvin *et al.*, 1958 a). ratio between potassium concentration index creatinine concentration index ($U/P_K/U/P_{Cr}$), on referred to as potassium excretion ratio, ects for increase in potassium concentration ndary to water abstraction. The excretion ratio eby provides information about the net move- out from or into the lumen, later on referred s net outflux and net influx respectively.

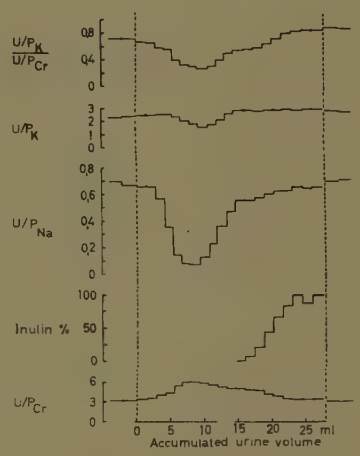


Fig. 1. Stop flow pattern on mannitol diuresis showing net potassium outflux from distal tubules without evidence of potassium secretion in spite of high rate of potassium excretion. Dog, weight 13.5 kg. Priming infusion: 0.5 g creatinine and 10 g mannitol in 100 ml water, followed by sustaining infusion of 2 g creatinine and 100 g mannitol in 1000 ml 0.9 per cent saline at 8 ml/min. After one hour for equilibration, ureteral catheter occluded for 6 min, followed by collection of serial urine samples. Concentration indices for creatinine, sodium and potassium, and potassium excretion ratio plotted from left to right against accumulated urine volume. Controls before clamping and 3 min after unclamping, plotted before and after the vertical broken lines respectively. Urine flow in control periods 10 ml/min. 5 ml 3 per cent inulin injected i. v. 45 sec before unclamping. Urine inulin concentration recorded in per cent of maximum value.

RESULTS

assium excretion pattern in experiments nannitol diuresis.

otassium excretion ratios from 0.4 to 1.0 e obtained during free flow. In about half he stop flow experiments the potassium etion ratios of all samples of urine ped in the distal part of the nephron e lower than free flow values. This ex- ion pattern without evidence of net in- in any segment was observed even when potassium excretion on free flow was as as 70 per cent of the filtered amount g. 1). The lowest potassium excretion was recorded in urine portions slightly imal to those having the lowest sodium centration. Potassium concentrations be- plasma level were obtained in 8 stop series, the lowest concentration index g 0.58. This provides unequivocal ence for active reabsorption of potassium. 12 stop flow series a distinct peak rred on the potassium excretion ratio e, localized slightly distally to maximal um reabsorption. On 6 occasions the exceeded 1; the highest recorded value was 1.7, providing unequivocal evidence for secretion of potassium. Proximal to this peak, but probably still in the distal tubule, the excretion ratio decreased below free flow values (Fig. 2). Potassium concentration of urine portions delayed in the proximal tubules was essentially equal to free flow values, as apparent from Figs. 1 and 2.

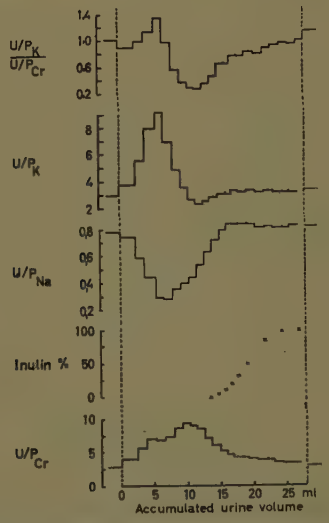


Fig. 2. Stop flow pattern on mannitol diuresis showing net potassium influx in distal nephron segments. Dog, weight 13 kg. 500 ml water by stomach tube and priming i. v. infusion of 1 g creatinine and 10 g mannitol in 100 ml water, followed by sustaining infusion of 2 g creatinine and 100 g mannitol in 1000 ml 3 per cent saline at 4 ml/min. After one hour the catheter was clamped for 9 min, followed by collection of serial urine samples. Urine flow in control periods 13 ml/min. 3 ml 3 per cent inulin injected i. v. 60 sec before unclamping.

In order to test the influence of anoxia on tubular potassium transport, the renal artery and vein were clamped during a stop flow period in two experiments. Net influx of potassium occurred in the distal part of the distal tubule, whereas the control series of these experiments obtained during free renal blood flow showed no evidence of net influx (Fig. 3). The net outflux of sodium from the distal tubules was reduced under anoxia.

Potassium excretion pattern in experiments on nonosmotic diuresis.

The main additional water reabsorption during stop flow experiments on water diuresis takes place in the ultimate distal pa of the nephron. Since this segment is loca ized distal to the sodium-diluting one, t water abstraction probably occurs in t collecting ducts where osmolar concentrati increase, mainly on account of the accumul tion of urea. A typical example is shown Fig. 4. The potassium concentration ind curve is bimodal. The distal increase is coe tensive with the accumulation of creatini whereas the proximal one is coexistent wi the large rise in sodium concentration. T

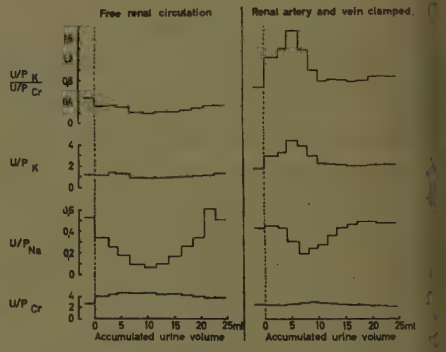
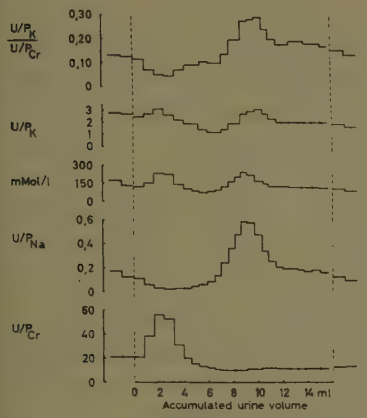


Fig. 3. Effect of clamping renal artery and ve Consecutive stop flow series performed on the sa kidney, showing less accumulation of fluid in distal tubules, reduced sodium reabsorption, high net potassium influx during the period obstructed renal circulation. Dog, weight 20 Priming infusion: 0.8 g creatinine, 10 g urea 10 g glucose in 50 ml water, followed by sustain infusion of 2 g creatinine, 200 g mannitol, 10 urea and 100 g glucose in 1000 ml 0.9 per saline at 8 ml/min. After approximately 45 for equilibration, ureteral catheter occluded 8 min, followed by collection of serial urine samp Diuresis in control periods 18 ml/min. 15 min l another occlusion of 8 min duration. Renal ar and vein clamped 15 sec after ureteral occlu and unclamped 15 sec before unclamping the ure Urine flow in control periods 22 ml/min. (Proxi urine portions not included in diagram.)



4. Stop flow pattern on water diuresis, show-
et outflux of potassium from collecting ducts
distal tubules. High potassium and sodium
ntration in portions following closely after
representing the distal tubules. Dog, weight
g. I v. infusion of 2 g creatinine in 1000 ml
per cent saline at 8 ml/min. In addition 700 ml
5 per cent saline was infused i. v. in the course
) min. After 1½ hour for equilibration the
ter was occluded for 6 min, followed by collec-
of serial urine samples. Urine flow: 2.8 and
ml/min in pre- and postperiods respectively.

mediate reduction in potassium concen-
on below free flow values evidently has
n place in portions delayed in the distal
les. The potassium excretion ratio, how-
, was in all experiments on water diu-
reduced below free flow values in all
l portions.

experiment where the osmolar concen-
on of urine on free flow is above plasma
s, the main creatinine accumulation dur-
stopped flow is coexistent with the
ction in sodium concentration as appa-
from Fig. 5. The main water reabsorp-
during stop flow on antidiuresis accord-
occurs in the distal tubules. In this
nent the highest potassium concentration

was reached. The potassium excretion ratio, however, was lowered relative to free flow values throughout the entire distal part of the nephron in all but 3 stop flow series, and reached minimum coextensive with or slightly proximal to the lowest sodium concentration (Fig. 5), whereas the minimum in experiments on water diuresis most frequently was reached in the collecting ducts (Fig. 4).

In 9 of a total of 23 stop flow series per-

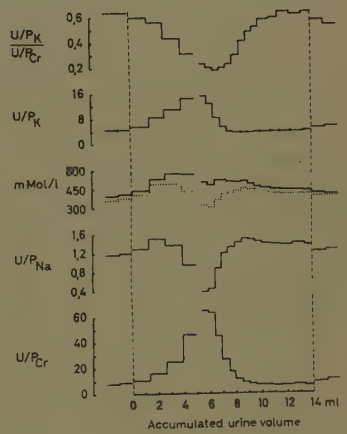


Fig. 5. Stop flow pattern on antidiuresis. Maximum net potassium outflux slightly proximal to the lowest sodium concentration. High potassium concentration in distal tubules due to water reabsorption, but no evidence of potassium secretion. Dog, weight 18 kg. Priming infusion of 0.5 g creatinine in 100 ml 0.45 per cent saline, followed by sustaining infusion of 2 g creatinine in 1000 ml 0.45 per cent saline at 8 ml/min. After one hour Pitressin was added to the sustaining infusion, 100 mU/ml, and 25 min later the ureteral catheter was clamped for 6 min, followed by collection of serial urine samples. Urine flow in control periods 6 ml/min. Osmolar concentrations calculated as 2 (Na + K) + creatinine + urea: ———, and 2 (Na + K) + creatinine: .....

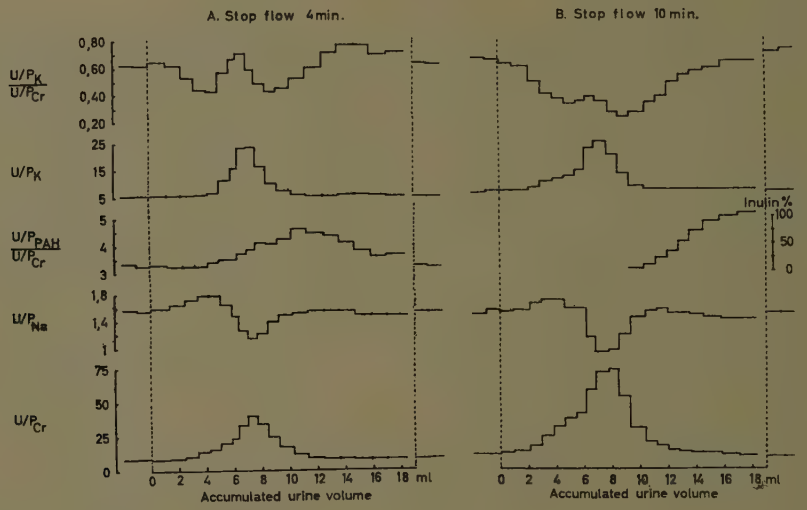


Fig. 6 a and b. Stop flow pattern obtained in consecutive periods of 4 and 10 min clamp-time, showing a) Net potassium outflux from collecting ducts and from proximal parts of distal tubules. Net influx in more distal parts of distal tubules. b) No further increase of distal tubular potassium contents, suggesting increased rate of potassium outflux during the last 6 min of occlusion. Dog, weight 18 kg. Priming infusion of 1 g creatinine and 100 mg PAH in 100 ml 3 per cent saline, followed by sustaining infusion of 2 g creatinine, 450 mg PA and 0.4 g KCl in 1000 ml 3 per cent saline 8 ml/min. After one hour a priming dose of 100 Diamox was given i. v. and 300 mg added to 1 ml sustaining infusion. 45 min later ureter clam for 4 min, followed by collection of serial u samples. Urine flow in control periods 7 ml/m After approximately 20 min, another occlusion 10 min duration. Urine flow 5.5 ml/min. 5 ml 3 cent inulin injected i. v. 45 sec before reinstatem of urine flow.

formed during nonosmotic diuresis, a distinct peak appeared on the potassium excretion ratio curve. As apparent from Fig. 6 a, this peak was localized slightly distal to the lowest sodium concentration. In only 3 experiments did the peak value exceed the potassium excretion ratio during free flow, and did not in any case exceed one.

It appears from these data that the potassium concentration in the lumen seems to be strongly influenced by the increased water reabsorption during stop flow on nonosmotic diuresis. If the increased concentration gradient for potassium over the tubular wall stimulates to diffusion out from tubule, or active reabsorption, it would reasonable to assume that the potassium c centration (or U/P_K) in the distal tubu would not increase appreciably in spite continued water abstraction, as when s flow experiment is prolonged.

In order to test this hypothesis, comp ison was made between two subsequent s flow series of 4 and 10 minutes durati The administration of Diamox in hyperto sodium chloride was continued through the experiment in order to induce potassi secretion (Pitts *et al.* 1958). The results

such experiment are apparent from . 6 a and b. The maximal potassium con- ration index for urine portions delayed he distal tubules is nearly equal after 4 10 minutes stop. The water abstraction , however, continued so that the creat- e concentration index has increased rly proportional to time. The distinct k on the potassium excretion ratio curve r 4 minutes stop flow has almost com- ely disappeared after 10 minutes occlu- , suggesting predominance of potassium lux in all distal nephron segments during last 6 minutes of occlusion.

DISCUSSION

he various factors regulating renal potas- n excretion are not well understood. The ular potassium content, acid-base equi- ium as well as hormones certainly part- ate in maintaining potassium home- sis. The reason for the different excre- patterns observed in different dogs even ing fairly well standardized experimental ditions (Figs. 1 and 2) can not be ex- ned by the parameters examined in the sent study.

slight increase in potassium excretion o from preperiods to clearance periods owing the stop flow series was observed many instances, but not invariably. In con- itive stop flow series almost identical retion patterns were obtained, indicating the potassium excretion is not seriously cted by the stop flow procedure *per se*. tubular processes visualized by the stop technique are therefore regarded as ng place also during free urine flow.

Tubular potassium secretion.

A potassium excretion ratio exceeding one necessarily implies net influx. In experiments on mannitol diuresis the peak value was invariably localized slightly distal to the lowest sodium concentration, as previously found by Malvin *et al.* (1958 a). Since a large fraction of the filtered load may be reabsorbed before the tubular contents reach the site of secretion of potassium, net influx of potassium may even be indicated by a peak on the excretion ratio curve not exceeding one. In several of the experiments on mannitol diuresis there was no evidence of a potassium secretion peak in spite of a potassium excretion ratio as high as 0.70 during free flow. This does not exclude the possibility of potassium influx during the stop flow period, but rather signifies that potassium outflux has prevalied in all nephron segments. It may therefore be concluded that in these cases of mannitol diuresis, the high rate of potassium excretion is due to failure of the tubules to reabsorb the filtered load rather than to a high rate of tubular secretion.

During occlusion of the renal artery and vein net influx of potassium markedly increased, possibly on account of impairment of the active reabsorption of potassium by the distal tubule. It is evident, however, that potassium influx may go on during anoxia of short duration. Changes in tubular functions produced by clamping the renal pedicle may not necessarily be due to anoxia alone. Accumulation of metabolites and change of pH may be of equal importance.

The interpretation of the stop flow pattern obtained when non-reabsorbable solutes have not been administered, is com-

plicated by the fact that tubular water reabsorption continues throughout the stop flow period. The slowing down of the flow-rate in the distal nephron segments, however, exaggerates the effects of tubular processes sufficiently to produce a clear localization pattern of the various processes. The continuous flow in the tubules during the stop flow period must, however, be taken into account when interpreting the results.

The potassium excretion ratio in experiments on nonosmotic diuresis did not exceed unity in any nephron segments in spite of excretion ratios as high as 0.95 during free flow. A peak on the curve for potassium excretion ratio was observed in several experiments localized slightly distal to the lowest sodium concentration, *i. e.* in the same segment where potassium secretion was demonstrated by mannitol diuresis stop flow. This peak might be due either to potassium influx or to a very low potassium outflux in this segment. The last possibility may be ruled out by the following reasoning: As apparent from Fig. 6 a, the main water reabsorption takes place in the distal tubule, but also in the segment where the potassium excretion ratio reaches its peak value. During the clamping period reabsorbed water is successively replaced by more proximal urine portions which evidently have a lower potassium excretion ratio than free flow urine. If only water was abstracted from these fluid portions on their way along the distal tubule, the absolute potassium concentration would increase, while the potassium excretion ratio would remain unchanged. Potassium outflux would reduce the excretion ratio, whereas an increasing excretion ratio, as seen in Fig. 6 a, must be due to potassium influx in this segment. The peak on the p tassium excretion ratio curve is therefo interpreted as being due to tubular potassiu secretion, without regard to the numeric height of the peak or its value relative the free flow excretion ratio. By this eviden potassium secretion was demonstrated du ing diuresis induced by Mercuhydri Diamox or large doses of Pitressin as w as during water diuresis, even when the p tassium excretion ratio on free flow was n higher than 0.12. Tubular potassium secr tion may accordingly contribute to t urinary potassium even when the potassiu excretion is low.

It has been widely accepted that t tubular potassium secretion consists of a exchange of potassium from the tubular cel with sodium from the tubular lumen (Be liner *et al.* 1950). This exchange is stimula ed by the presence of non-reabsorbable anio and probably plays a minor role under no physiologic conditions. In our experiment net potassium influx was regularly localize distal to maximal sodium outflux, and w demonstrated also in the presence of lo sodium concentration in the distal tubul (Fig. 2). This is in agreement with the fin ings of Anderson & Laragh (1958), wh found identical potassium excretion aft potassium loading in sodium depleted do and in normal controls.

Tubular potassium reabsorption

Distal tubule. Net outflux of potassium the distal tubules was shown to take pla in stop flow experiments both during man itol and nonosmotic diuresis. The lowest p tassium excretion ratio was regularly recor ed in urine portions slightly proximal

se having the lowest sodium excretion o. This might suggest a different local- ion of potassium and sodium reabsorp- . As mentioned above, potassium influx es place mainly in the distal part of the al tubule, and the apparently different lization of net potassium and sodium out- might thus be explained. The hypothesis a common reabsorptive mechanism for ium and potassium (Koch, Brazeau & nan, 1956) is therefore not excluded by present experiments.

n several experiments the potassium con- tration of distal urine portions was be- plasma levels. Although the potassium centration of the cortical interstitial water ot known, it is probably not lower than of plasma. Potassium is accordingly oved from the distal tubule against a centration gradient, implying active sport of potassium by the tubular cells. he rate of outflux from the distal tubules lently increases with increasing intra- inal potassium concentration. As seen n Fig. 6 a and b, the potassium contents he distal tubule did not increase appreci- from the fourth to the tenth minute of ping. Further net influx has accordingly taken place. It may be calculated from creatinine concentration index curves the rate of distal water reabsorp- has been roughly unchanged from the four to the last six minutes of occlusion. llows that the rate of potassium delivery ne distal tubules has also remained nearly stant. Since the potassium contents of the al tubules did not increase, the rate of lux must have been higher during the six minutes of occlusion. As the po- ium excretion ratio is virtually unaltered between pre- and post-periods, it seems reasonable to suggest that the increase of net potassium outflux during the last 6 minutes is due to the high absolute potassium concentration established in the distal tubules during the first minutes of stop flow.

Recently Jaenicke & Berliner (1960) suggested that the low potassium concentration of distal urine portions observed during mannitol diuresis stop flow is not due to potassium reabsorption in this segment. The following explanation was proposed: Potassium is added to the urine in the collecting ducts and the distal tubule in exchange with sodium. Urine portions arrested in the distal tubules contain small amounts of sodium for this exchange process, and when these urine portions pass the site of exchange after release of stop, little potassium is added. This explanation is not compatible with the present finding. In Fig. 6, marked lowering of the potassium excretion ratio is observed in distal urine portions in spite of high sodium concentration. Furthermore, the lowest concentration in mannitol stop flow was regularly observed proximal to the sodium minimum while, according to the "out-flow hypothesis" of Jaenicke & Berliner (1960), the sodium and potassium minima should be coextensive.

Collecting ducts. In several experiments performed during mannitol diuresis, the potassium excretion ratio was already lowered, relative to free flow values in the first urine portions collected, suggesting potassium outflux also from the collecting tubules (Fig. 1). This might, however, as well be due to admixture of urine arrested in the distal tubules. Furthermore, the collecting ducts can not be exactly defined in the stop flow pattern of

mannitol diuresis. In some experiments performed during water diuresis, however, the lowest potassium excretion ratio was recorded in urine portions arrested in the collecting ducts (Fig. 4). The decrease of potassium excretion ratio from the distal tubule to the collecting ducts is probably the result of net potassium outflux from the collecting ducts. Potassium concentrations below plasma levels were not measured in urine portions from the collecting ducts. The outflux therefore does not necessarily imply active reabsorption of potassium from this segment. During stop flow experiments on water diuresis the main additional water abstraction takes place in the collecting ducts with ensuing increase in intraluminal potassium concentration. Since potassium outflux is most readily demonstrated during these experimental conditions, this constitutes additional evidence for the hypothesis that potassium outflux increases with increasing intraluminal concentration.

In a previous study (Kiil & Aukland 1960 b) evidence was presented supporting the hypothesis that the distal part of the collecting ducts is freely permeable to urea so that an equilibrium is established between collecting ducts and papillary interstitial water where urea is trapped to a large extent. During the sampling following stopped flow, urea diffuses into the papilla from urine portions of high urea concentration. In subsequent urine portions of low urea concentration, urea will diffuse back from the papilla so that the urea excretion ratio for these portions might exceed unity. (This unique mechanism of urea is the reason why a presumably more correct osmolar excretion pattern is obtained during antidiuresis when the osmolar concentration of urea is sub tracted from the total osmolar concentratio (Fig. 5).) Since potassium outflux increase with increasing intraluminal potassium con centration, this might suggest that the col lecting ducts also were highly permeable t potassium and that potassium played a rol similar to that of urea in the urine-concen trating mechanism. The outflux patteren gave no support for this hypothesis.

Low excretion ratios for potassium in th collecting ducts were not invariably found presumably on account of the confoundin effect of potassium secretion, which possibl may tak place in the same segment. Th variable findings concerning the fate of po tassium in the collecting ducts are in agree ment with the results of the microcatheter ization studies of Hilger, Klümper & Ull rich (1958).

Analysis of proximal stopflow pattern

The analysis of proximal tubular function is complicated by the fact that urine portion which have been arrested in the proxima tubules have to pass through the rest of th nephron, including the loops of Henle, befor being collected. In stop flow experiments o mannitol diuresis, the absolute potassiu concentration on free flow was maintaine practically unaltered throughout the prox imal tubule (Figs. 1 and 2). It has bee argued by Malvin, Wilde, Vander & Su livan (1958 b) that unchanged sodium co centration means isotonic reabsorption i the proximal tubules without active hand ling of sodium. With regard to potassiu this statement disagrees with the results Wirz & Bott (1954) who, by micropunctio studies in the rat, found decreasing pota

concentration down the proximal tubu- A possible explanation might be that ssium is actively reabsorbed by the prox- tubules, whereas back diffusion of potas-, proportional with the gradient across ubular wall, reduces net potassium out- from the proximal tubules. If equi- um between active reabsorption and back sion is reached already during free man- diuresis, no further reduction of the ssium concentration in the proximal les would be expected. The same me- ism might also explain unchanged prox- sodium concentration during mannitol flow.

most experiments performed on non- otic diuresis, the potassium concentration steeply in urine portions following im- ately after those representing the distal le. A similar pattern has been recorded odium (Kiil & Aukland 1960 a) and for nesium. The increase in osmolar concen- on of urine remaining in the collecting s reflects a similar increase in osmolar entration of the interstitial water of the lla. The surplus of osmolar constituents ashed out during the sampling period, the osmolar concentration of urine in periods equals that of preperiods. The ased concentration of cations in prox- fluid portions is assumed to be due to shout of a high concentration of these in the renal medulla. Whereas there is ge difference in sodium concentration of llary tissue water between antidiuresis water diuresis (Ullrich & Jarausch), the failure of finding significant dif- ces in potassium concentration may be o the mainly intracellular localization of sium. It therefore seems justifiable to suggest that potassium reabsorption from the ascending limb of Henle's loop (which probably is included in the stop flow concept distal tubules) participates in the mechanism of urine concentration, although the quantitative significance of this potassium transport is small.

SUMMARY

1. The stop flow excretion patterns for potassium were studied during various types of diuresis, induced by mannitol, water or hypertonic sodium chloride or by the administration of Mercuhydrin, Diamox or Pitressin. In all, 52 stop flow series were performed in 25 dogs.

2. Independent of the type of diuresis, tubular potassium secretion was found to be localized slightly distal to the maximal reduction in sodium concentration. Net influx into the tubular lumen was present during anoxia of short duration.

3. In the stop flow pattern the lowest proximal to the lowest sodium concentration. In several experiments the potassium concentration of these portions was less than that of plasma, indicating active distal reabsortion. The rate of net potassium outflux increased at high intraluminal potassium concentration.

4. Potassium outflux from the collecting ducts (defined as the segment of increased osmolar concentration in the stop flow pattern) was demonstrated in several experiments, especially during water diuresis.

5. In several stop flow patterns, also in those obtained after mannitol loading, net outflux of potassium prevailed in all segments without evidence of potassium secretion. The high rate of potassium excretion

during mannitol diuresis is probably due to failure of potassium reabsorption rather than to a high rate of tubular secretion.

6. In stop flow patterns where the osmolar concentration increased in the collecting ducts, proximal portions were of high sodium and potassium concentration, presumably due to washout of a high concentration of these ions in the renal medulla.

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SIMULTANEOUS DIRECT AND INDIRECT INULIN CLEARANCE AS A SEPARATE KIDNEY FUNCTION TEST

By P. LEYSSAC and J. LUND

From Surgical Department A and The Laboratory, Copenhagen county hospital, Gentofte, Denmark

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Methods for evaluating the function of each kidney separately are of value particularly in diagnosing unilateral surgical and medical renal diseases, but also in deciding the extent of a surgical procedure. In most previous investigations bilateral ureteral catheterization has been used (Chasis and Redish 1941, Schroeder 1954, Larsen 1945, Kronik 1952, Nesbitt 1954, Edvall 1958, and Haugen, Høeg, Vogt & Blegen 1960). The most serious source of error in this method is oozing of urine beside the catheters, an error which excludes clearance tests. By simultaneous collection of bladder urine, it may be calculated from the concentrations in the bladder and in the ureters, how large a proportion of the bladder urine is derived from the right and how large a proportion from the left kidney in the event of a difference in the concentration on the two sides (Larsen 1945, Kronik 1952). Using suitable catheters several authors have succeeded in collecting ureteral urine quantitatively (Chasis and Redish 1942, Keutel 1954, Nesbitt 1954, Edvall 1958 a, b, and Haugen *et al.* 1960).

A special problem is presented by hydronephrosis, since the ureteral urine on the side of the hydronephrosis does not always reflect the renal function. The composition of the urine will be altered by the admixture of urine of unknown composition retained in hydronephrotic sac. The function of th kidney may also be altered, if a ureter catheter is carried past the obstruction, thu relieving the hydronephrosis (Edvall 1959)

The present method for determining th glomerular filtration rate of each kidne separately was designed for use in case where the ureteral urine on one side is in applicable for clearance determination. It principle is determination of the clearanc indirectly, using a constant infusion tech nique by the Pers modification of Erle an Berliner's method (1953). With this metho the infused quantity is equal to that ex creted during the same period, when th plasma concentration remains constant. Th clearance may, thus, be calculated as the in fused quantity of inulin per minute divide by the plasma concentration. At the sam time, the clearance of one kidney is dete mined directly as the excretory clearance o ureteral urine. Then, the clearance of th other kidney may be determined as th difference between the total clearance an the clearance of the directly tested kidney A statistical evaluation of the uncertainty clearance determination in Pers modificatio of the indirect method does not appear t have been reported previously.

METHOD AND MATERIAL

a measure of the glomerular filtration rate, clearance was used.

e direct excretory clearance, measured on er urine (total excretory clearance) or on al urine (unilateral excretory clearance) was ated on the basis of the usual formula UV/P where U is the concentration in the V the urinary output per minute, and P the a concentration. As already mentioned, the ct total clearance was determined using a ant infusion technique by the method of Pers. the first 5 experiments the total glomerular ion alone was determined, partly by collecting er urine during 3 successive periods and partly etermining simultaneously the indirect total nce by the Pers infusion technique. In the quent 6 experiments ureteral urine was col- from both sides simultaneously with constant on for indirect clearance determination.

e procedure was as follows: After a light ugar breakfast with ample tea, a priming dose out 10 g inulin was administered by the in- nous route. Thereupon, ureteral catheters tle-tip catheters with many holes No. 5—7) introduced about 15 cm into each ureter. In on, an indwelling catheter was inserted into ladder.

) min. after the priming dose, a continuous on of about 3 per cent inulin solution was d, using an infusion apparatus of the same as that used by Pers (1953). The apparatus djusted to a constant infusion rate of 30 ml/ corresponding to about 16 mg inulin per min. e end of 10—15 minutes, the collection periods started.

ior to each collection the bladder was rinsed saline solution and emptied by instilling 20—30 r, exerting light suprapubic compression dur- ie emptying process. Then 100 ml saline solu- was immediately instilled into the bladder, and reteral catheter was clamped off. Immediately the collections of urine were started. After period, the contents of the bladder were ed out, measured and analysed in order to how much had oozed out beside the ureteral ters during each period.

eteral urine was collected during 3 successive periods of 10—20 min. each. In the middle of each period a venous blood sample was removed for determining the plasma inulin concentration.

After the experiments were completed the patients were given opio-papaverine, 1 ml, or pethidine, 1.5 ml (75 mg) subcutaneously in order to prevent or alleviate colic. During the first 24 hours, furadantin or sulphamethizole were given prophylactically in the usual therapeutic doses.

In each experiment the infused inulin solution was analysed in order to determine accurately the infused amount per time unit.

Inulin analysis was carried out by Bojesen's modification of the diphenylamine method (1952), and the analyses were performed in duplicate.

The material comprised women admitted in most cases for diseases which could not have affected their renal function. Two of the patients had unilateral pyelitis (Experiments k and m, Table III).

RESULTS

A. *Comparison of total clearance simultaneously determined, indirectly by the method of Pers and directly in bladder urine.*

The clearance values listed are mean values of 3 successive clearance periods corrected to 1.73 m^2 surface.

Table I shows the results of total clearance in 5 experiments measured as direct excretory clearance on bladder urine and simultaneously as indirect clearance by the method of Pers. It will be seen that the "classical method" of collecting bladder urine carries considerable inaccuracy, an inaccuracy so marked that the range within which 95 per cent of the observations will be situated (twice the e_M to each side) is 18 ml/min./1.73 m^2. The Pers method, on the other hand, gives a considerably less marked dispersion of the results, provided that the plasma concentration is constant.

The difference between the mean values of

Table I. *The total Inulin clearance, determined by the direct method (bladder urine) and an indirect method (the Pers modification).*

No.	Total excretory clear. from bladder urine (ml/min/1.73 m²)	Variance s^2	Total indirect clear. a. m. Pers (ml/min/1.73 m²)	Variance s^2	Difference between direct and indirect clearance (ml/min/1.73 m²)
1	45.7	46.6	42.9	1.5	+ 2.8
2	64.7	11.1	60.6	0.3	+ 4.1
3	117.7	86.3	126.0	0.6	— 8.3
4	115.5	157.0	127.9	8.0	—12.4
5	119.9	6.2	124.2	x	—12.3
	Σs^2 : 307.2		Σs^2: 10.4	mean: — 5.22	
	SD** : 7.84		SD : 1.61	SD : 8.1	
	eM***: 4.52		eM: 0.93	eM : 3.62	
				t : 1.44	

*: one determination only,
**: standard deviation (ml/min/1.73 m²),
***: standard error of the mean (ml/min/1.73 m²).

total excretory clearance on bladder urine and total clearance by the method of Pers was calculated in each experiment, and the t value was found to be 1.44 (< 2.5). In other words, there is not a significant difference between the two methods for determining total clearance.

B. *Comparison of total clearance simultaneously determined, indirectly by the method of Pers, and directly as the sum of the excretory clearance of the right and left kidneys.*

Table II gives the total clearance, partly calculated as the sum of the excretory clearance of the right and left kidneys determined directly on ureteral urine and partly the indirect clearance simultaneously determined by the method of Pers. From these values it will be seen that the total excretory clearance determined on collected ureteral urine has a mean inaccuracy of the sa magnitude as the total excretory clearan on bladder urine (Table I). The indir method carries less inaccuracy. The me error in the indirect clearance determinati by the method of Pers in all the 11 expe ments (Tables I + II) was 1.17 ml/mi 1.73 m². Calculating the difference betwe the methods of evaluating the total clearan (Table II), t values of 0.54 were found. other words, there was no difference betwe the two methods.

C. *Comparison of directly determined and calculated unilateral clearance.*

Table III gives the unilateral clearan values. It will be seen that the mean er in the direct unilateral clearance on urete urine is 2.43 ml/min./1.73 m². As rega the computed unilateral clearance, calcula from the total indirect clearance determi

Table II. *The total Inulin clearance, determined by the direct method (urine from right and left ureter) and an indirect method (the Pers modification).*

	Total excretory clear. (r. plus l. urete (ml/min/1.73 m²)	Variance s^2	Total indirect clear. a. m. Pers (ml/min/1.73 m²)	Variance s^2	Difference between direct and indirect clear. (ml/min/1.73 m²)
f	115.7	312.1	120.2	9.3	— 4.5
g	99.3	0.3	97.5	9.3	+ 1.8
j	76.7	95.6	79.8	0.3	— 3.1
k	106.1	74.0	107.5	0.4	— 1.4
n	101.8	27.1	95.4	1.9	+ 6.4
n	105.6	19.9	98.2	10.1	+ 7.4
	Σs^2 : 528.9		Σs^2: 31.3	mean: + 1.1	
	SD* : 9.39		SD: 2.28	SD : 4.97	
	eM** : 5.42		eM: 1.32	eM : 2.03	
				t: 0.54	

SD: standard deviation (ml/min/1.73 m²).
eM: standard error of the mean (ml/min/1.73 m²).

Table III. *The unilateral Inulin clearance, determined by the direct method (ureteral urine) and calculated as the difference between simultaneous total clearance a. m. Pers and contralateral, excretory, unilateral clearance.*

	Unilateral, direct clear. ureteral urine. (ml/min/1.73 m²)	Variance s^2	Unilateral calculated clear. (ml/min/1.73 m²)	Variance s^2	Difference between unilat. direct. and unilat. calculated clear. (ml/min/1.73 m²)
f	52.7 (r)	14.6	63.8	16.5	— 4.6
	56.4 (l)	38.0	61.0	91.5	— 4.6
g	52.7 (r)	0.41	50.9	8.0	+ 1.8
	46.6 (l)	0.1	44.7	13.5	+ 1.9
j	41.6 (r)	15.4	48.0	26.9	— 6.4
	35.1 (l)	19.3	38.2	13.4	— 3.1
k	64.1 (r)	20.4	65.5	15.5	— 1.4
	42.1 (l)	20.9	43.5	15.2	— 1.4
n	39.6 (r)	18.9	33.6	50.8	+ 6.0
	61.8 (l)	47.3	55.5	11.1	+ 6.3
n	58.2 (r)	0.1	50.9	3.9	+ 7.3
	47.3 (l)	19.5	40.0	10.3	+ 7.3
	Σs^2 : 214.9		Σs^2: 276.8	mean: + 0.76	
	SD* : 4.20		SD : 4.8	SD : + 5.03	
	eM** : 2.43		eM : 2,77	eM : + 1.45	
				t: 0.52	

SD: standard deviation (ml/min/1.73 m²).
eM: standard error of the mean (ml/min/1.73 m²).

simultaneously by the Pers method and the excretory clearance of the contralateral kidney determined on ureteral urine, the mean error was found to be 2.77 ml/min./1.73 m². Thus, the inaccuracy of the two methods for determining unilateral clearance is of the same magnitude. Calculation of the difference between the two methods for assessing the function of one kidney separately on the basis of the mean values of the unilateral clearance for each method in each experiment, revealed no significant difference between the two methods, the t value being 0.52.

In experiments k and m, there was a slightly impaired renal function of the left and right kidney respectively, assessed on the basis of inulin clearance. In both experiments, the functional impairment accorded with the clinical signs, the patient of experiment k having acute pyelitis on the left and the patient in experiment m acute recurrent pyelitis on the right.

In all the experiments the urinary output decreased, after the ureteral catheterization, from about 10 ml per min. to about 2 ml min. The inulin clearance was not altered by the catheterization.

In 5 of the 6 experiments the collection of ureteral urine was quantitative, there being only traces of inulin in the bladder urine. In one case between 1 and 3 ml had oozed out beside the catheter in each collection period.

The plasma concentration of inulin following continuous infusion of about 16 mg per min., as described above, remained constant in all the experiments. This is in keeping with the results of Pers (1953).

In 10 analyses on the same per cent inulin solution the analytical error did not exce 3 per cent.

Complications.

Of a total of 13 patients submitted ureteral catheterization, 11 developed hem uria. In only one case, however, was it seve enough to occlude the catheters.

About half the patients (a total of developed pain or discomfort, but only 2 severe that the experiment had to be int rupted. The pain rapidly yielded to op papaverine. Five did not feel any disco fort at all.

There was no case of ascending urin tract infection after the experiments.

DISCUSSION

Seven of our first experiments fail because the ureteral catheter on one s slid down into the bladder again or beca the catheters could not be introduced on or on both sides. In one case the cathet became occluded by a clot, and the exp ment had to be stopped because of colic. must be emphasized that most of the f ures occurred in the beginning, before had acquired the necessary routine in troducing the fairly thick catheters.

The risk of oozing of urine beside catheters may presumably be reduced by ducing osmotic diuresis, whereby the ex of the error will also be diminished. Possi an increased urinary output may also red the risk of clot formation and thereby of colic.

Due to the large quantities of cont medium used nowadays, intravenous graphy has lost its value as a kidney funct

since the plasma concentration of con-
t medium by far exceeds the limit of self-
ression. Thus, the contrast in the renal
is may easily be the result of glomerular
ation alone. As a function test, in-
enous urography is probably not superior
he determination of blood urea (Edvall
8a).

ssessment of unilateral kidney function
enal vein catheterization and determina-
of the extraction ratio is unsuited for
purpose for which the present method
worked out. The fact is that in hypo-
tic kidneys and kidneys with compen-
ry hyperplasia, there is a less satis-
ory correlation between the extraction
and the excretory clearance (Edvall
8b). Ole Munck (1958) also found that
l vein catheterization determining the
riovenous deficit of a substance was
licable only in the presence of good
lar function, *i. e.* when the arteriovenous
cit is sufficiently great. Otherwise, the
rmination will be too inaccurate, and this
applies to the calculation of RBF_{PAH}
he direct method of Fick.

he present method permits the deter-
ation of the unilateral inulin clearance
on a kidney from which the ureteral
e is inapplicable for clearance determina-
. The inaccuracy of the clearance deter-
ation is of the same magnitude as the
ct clearance determination measured on
eral urine, and unilateral clearance may
letermined with an inaccuracy only half
nat attaching to total clearance determin-
on bladder urine. The fairly marked in-
racy found in clearance determination
ladder urine is presumably due to an in-
cient emptying of the bladder by an
indwelling inflatable catheter. As a rule about 5 ml remained in the bladder, presumably because the tip of the inflated catheter is a little above the base of the bladder when the balloon is filled.

SUMMARY AND CONCLUSION

A comparison of simultaneous direct and indirect inulin clearance determination of total renal function as well as unilateral function gave the following results:

(*A*). When the clearance is expressed as the mean value of 3 successive determinations, statistical analysis of the results showed that the mean error in determining the total clearance on bladder urine ("classical method") was 4.5 ml/min./1.73 m², whereas the total clearance determined indirectly using a constant infusion technique, had a mean error of only 1.2 ml/min./1.73 m², when the plasma concentration remained constant. There was no significant difference between the mean values of total clearance determined by these two methods.

(*B*). Determination of the total excretory clearance as the sum of the excretory clearance of the right and the left kidneys carried a mean error of the same order of magnitude as determination on bladder urine. The sum of the clearance of the right and the left kidneys did not differ significantly from the results of simultaneous determination of the clearance by an infusion technique.

(*C*). By determining the total clearance by the constant infusion technique and simultaneously the clearance of one kidney as direct excretory clearance on collected ureteral urine, the clearance of the other kidney was determined as the difference between the

two values. Directly determined unilateral excretory clearance may be determined with a mean error of 2.4 ml/min./1.73 m². If the unilateral clearance is determined as the difference between the indirect total clearance and the clearance of the opposite kidney, the mean error is 2.8 ml/min./1.73 m². There was no significant difference between the mean values of unilateral clearance determined by these two methods.

It is concluded that the method is applicable in cases where the ureteral urine from one kidney cannot be collected or is inapplicable for clearance determination.

Owing to the cumbersome technique and the special apparatus, the method is not suited for ordinary clinical use.

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EFFECT OF ESTROGENS ON THE HAPTOGLOBIN LEVEL IN THE BLOOD

By N. E. BORGLIN and M. NYMAN

From the Department of Obstetrics and Gynecology and the Departmend of Clinical Chemistry, University of Lund, General Hospital, Malmö, Sweden

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ıe concentration of serum haptoglobin
/s), a protein belonging to the electro-
etic alpha$_2$ fraction, is known to vary
ly in different pathologic states. It in-
ses in connection with inflammatory re-
ns and tissue proliferation and decreases
bjects with an increased red cell catabol-
and with parenchymatous liver disease
references, see Nyman 1959).
ırlier studies dealing with the effect of
ogens on different serum proteins have
n that the Hp level is to some extent
ected to hormonal regulation, as it was
d to increase regularly after administra-
of androgens to women (v. Studnitz &
ıan 1957).
ıe present paper is a study of the varia-
of the blood Hp level in healthy women
administration of estrogens.

MATERIAL

roup I. Fifty-nine apparently healthy women
menopause (43—81 years), who were admitted
clinic of gynecology for surgical treatment
rine and vaginal prolapse.
of them had a quite normal hemoglobin level
ıe ESR showed no appreciable increase. These
n were treated with estrogens during a period
-7 days before surgical intervention to secure
healing conditions (Sjöstedt 1953).

Blood analyses as well as other determinations were performed before the treatment had begun, and repeated 1—4 times in the course of it.

In 18 of these 59 cases the endometrium was curetted during the observation period. In order to preclude any influence of this operation on the Hp level, Hp determinations were also performed before and after curettage in 9 women to whom no estrogen therapy was given.

Group II. Four healthy women in the fertile age (21—38 years) with primary or secondary amenorrhoea. They were treated with estrogens monthly for periods of 3 weeks. Two of them were also given 17-α-methyl-19-nortestosterone (Orgasterone®) during the third week of estrogen treatment. During the 4th week all therapy was withdrawn. Blood analyses and other determinations were performed twice a week during one month.

Group III. Estrogens were given to one healthy woman (37 years), without any symptoms from the genital organs, for two periods of 5 days with an interval of one month. Blood analyses and other determinations were performed daily during treatment as well as some days before and afterwards.

Estrogenic substances used

a) *Diethylstilbestrol* was given orally to 32 women of group I and to the woman in group III in doses of 3 mg a day.

b) *Estriol* (Ovesterin®) was given intramuscularly to 27 women of group I in a dose of 1 mg a day.

c) *Ethinylestradiol* (Linoral®) was given orally to the women in group II in a dose of 0.05 mg a day.

METHODS

Hp determinations 1) according to Jayle (1951)[1]
2) according to Nyman (1959)

According to Jayle (1951), the peroxidatic activity of the HbHp complex is measured. Nyman (1959) gives a direct measures of the amount of HbHp complex formed.

Paper electrophoresis of serum proteins according to Laurell, Laurell & Skoog (1956).

Coeruloplasmin determinations with a modified Ravin (1956) technique.

Carboxyhemoglobin (CoHb) determinations were kindly performed by Gydell with a modification of the method of Linderholm & Sjöstrand (1956).

According to Sjöstrand (1952), Hallberg (1955) and Engstedt (1957), the carbon monoxide level in the blood gives a realiable measure of the amount of hemoglobin catabolished, as 4 molecules of CO are liberated for each molecule Hb that is broken down. According to these authors, there is no other endogenous source of carbon monoxide in humans.

Total reticulocyte count of the blood according to Björkman (1958).

Osmotic resistance of red cells.

Determination of coagulation factors.

Fibrinogen, prothrombin (P and P), factor V, prothrombin consumption and plasmine (lysed area in mm²) were kindly determined by I. M. Nilsson.

RESULTS

A) *Influence of the addition of diethylstilbestrol and estriol in vitro on the serum Hp concentration.*

A solution of diethylstilbestrol in ethanol (concentration 40 mg/100 ml) or of estriol in ethanol (concentration 35 mg/100 ml) was added to different samples of one and the same serum. The Hp concentration was titrated after an incubation of 1 hour at room temperature. The same values were obtained in the estrogen enriched sera as in the co trol serum.

The estrogen concentration in the sampl was about 0.4 mg/100 ml, *i. e.* an amou greatly exceeding the concentrations of the preparations in the blood after oral or *i.* administrations of the doses as given in th investigation.

It is thus clear that the estrogens used the investigation had no effect on the H value recorded when added to serum *in vitr*

B) *Variations of the Hp level during or administration of diethylstilbestrol to healt women. (N = 33, from groups I and III*

The difference of the actual Hp value fro the original level (in mg/100 ml) has be

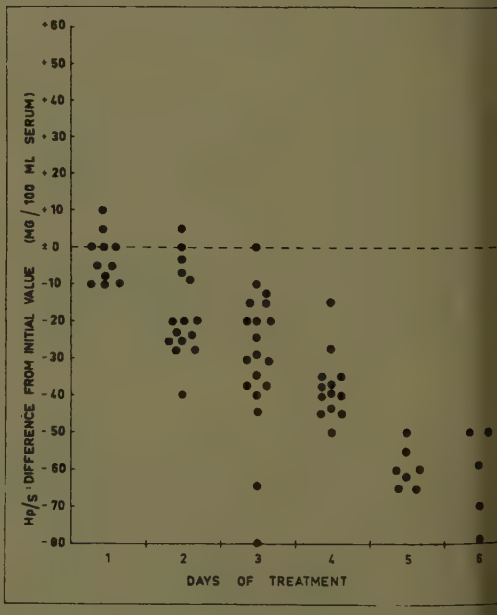
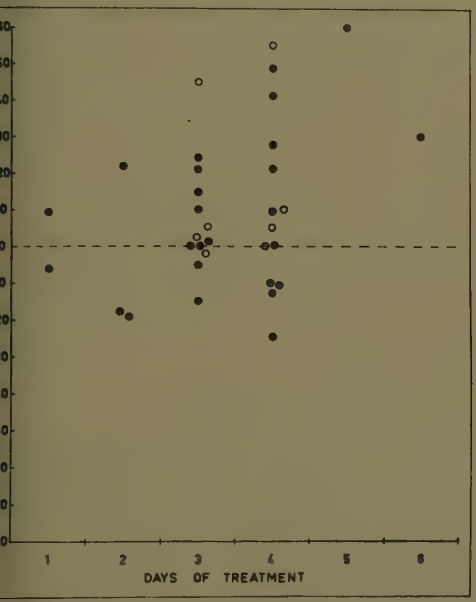


Fig. 1. Variations of the Hp level in the blood d ing oral administration of diethylstilbestrol to healthy women (1—4 values per case).

[1] When not further specified Jayle's method was used throughout this investigation.

ted to the duration of the treatment
ys) (2—4 samples per case have been
rded and are reported in Fig. 1).
n all cases treated for more than 2 days,
ecrease in Hp concentration was noted
this decrease was related to the total
unt of stilbestrol that had been orally
inistered during the observation period
ax. 6 days).
he Hp values recorded after treatment
e identical whether the determination was
formed according to Jayle (1951) or Ny-
n (1959). This implies that the decrease
he Hp values was due either to a decrease
the hemoglobin binding capacity of the
toglobins or in the haptoglobin concen-
ion, and thus was not a methodological
fact.



2. Variations of the Hp level in the blood
ng i. m. administration of estriol to 27 healthy
women.
estriol ⊙ estriol + curettage O curettage.

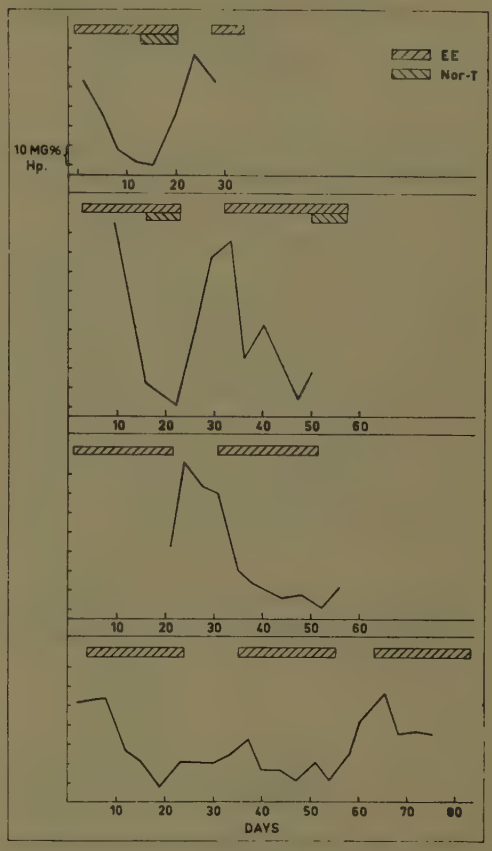


Fig. 3. Variations of the Hp level in the blood during and after oral administration of ethinylestradiol to 4 women
EE: ethinylestradiol
Nor-T: 17-α-methyl-19-nortestosterone
(Each interval on the ordinate represents an amount of 10 mg Hp per 100 ml.)

C) *Variations of Hp/s level during administration of estriol to healthy women (N = 27, from group I).*

The data are recorded in Fig. 2. There were no regular changes of the Hp/s level in connection with estriol treatment. The Hp concentration of the controls, who had been

curetted without receiving any estriol, showed the same variations as the rest of the material.

D) *Variations of the Hp level during administration of ethinylestradiol (Linoral®) (N = 4, group II).*

As seen in Fig. 3 this natural estrogen compound had the same effect on the Hp concentration in the serum as the synthetic form diethylstilbestrol. These figures also show that the rapid decrease of the Hp concentration was limited to the first week of therapy, and that the values then successively stabilized on a low level. As soon as the drug was withdrawn there was a rapid increase in the Hp level, especially in the 2 women to whom 17-α-methyl-19-nortestosterone was given during the last week of estrogen therapy.

E) *Variations of other serum proteins du ing administration of diethylstilbestrol.*

Two samples of serum (one collected b fore and the other at the end of the observ tion period) from each of 20 cases fro group I were analyzed with the aid of pap electrophoresis. In 7 of the cases coerul plasmin activity was also estimated. T variations of the different protein fractio are recorded on Fig. 4.

There was thus a decrease in the albun and gamma-globulin level — about 5— per cent — and an increase of the alph globulins fraction as well as of the "n haptoglobin" alpha$_2$ globulins, while the lev of the beta globulins seemed to be unaffecte The increase of the coeruloplasmin conce tration which has already been noted

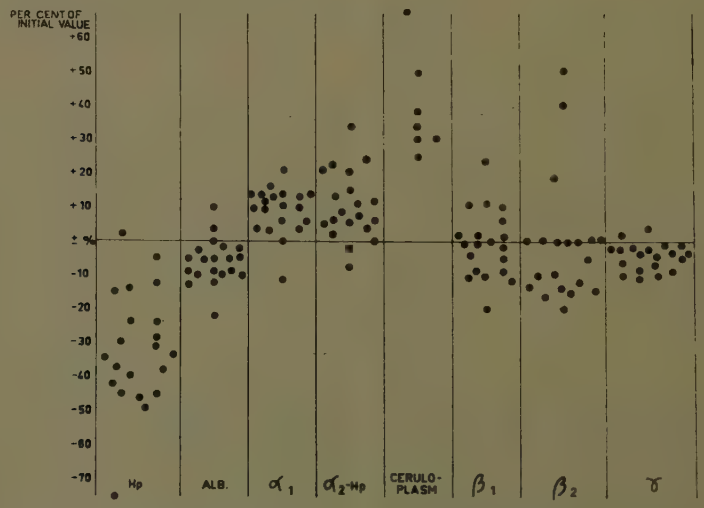


Fig. 4. Variations of the main serum protein fractions and of coeruloplasmin level in the blood during oral administration of diethylstilbestrol to 20 healthy women.

ezin & v. Studnitz (1957) is not suf-
nt to explain the increase in the alpha$_2$
ion.

Effect of diethylstilbestrol and ethinyl-
adiol on the turnover of erythrocytes.

Ve have tried to evaluate the red cell
luction by counting circulating reticulo-
s, and the red cell catabolism with the
rect method for estimation of endogen-
CO formation.

here was no appreciable change in the
ber of circulating reticulocytes whether
e were evaluated in percentage of circu-
g red cells (20 cases) or as the total
ber of reticulocytes per 0.001 ml blood
ases).

ne results of repeated determinations of
COHb level (2—5 per case) in 16 women
ed with diethylstilbestrol are reported
ig. 5. An increase was noted in most of
e cases. It reached its maximum 24 hours
the start of treatment. The values
ed to stabilize on this level for 2 or 3
and then slowly to decrease again.
statistical analysis of the data recorded
ig. 5 is given below.

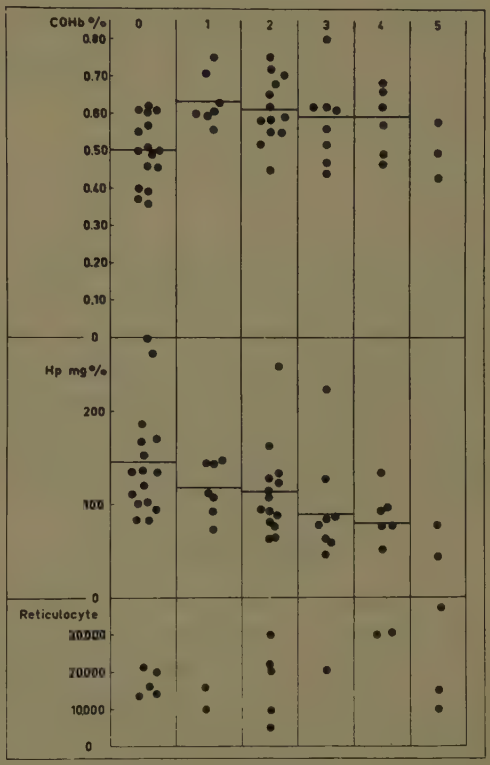


Fig. 5. Variations of the Hp level (mg per 100 ml serum) to COHb level (COHb per centage) and the reticulocytes (reticulocyte count in 1 mm^3 of blood) during oral administration of diethylstilbestrol. Abscissa: days of treatment.

ificance of difference between the COHb
es recorded on different days of estrogen
apy and the original COHb level (= 0).

of treatment	0-1	0-2	0-(3-4-5)
ificance	++	++	++

$= 0.1$ per cent $< P < 1$

4 cases treated with estriol for 4 and
ys there was no increase of the COHb level found on daily determinations during treatment.

The osmotic resistance of the erythrocytes was determined in 9 cases before and at the end of the observation period. No changes were recorded.

G) *Effect of diethylstilbestrol treatment on the serum transaminase level (GPT).*

The glutamic pyruvic transaminase was recorded in 1 case (group III) before and

during the treatment with diethylstilbestrol (6 determinations). The level was normal and unchanged during the whole observation period.

H) *Effect of diethylstilbestrol on blood coagulation factors.*

Determination of bleeding time, coagulation time, prothrombin (P and P), factor V, prothrombin consumption, plasmine and fibrinogen before and during treatment with diethylstilbestrol (5 determinations on successive days) showed no changes in any of these coagulation factors.

DISCUSSION

The studies reported above have clearly shown that oral administration of two different estrogens — the substituted natural form ethinylestradiol and the synthetic diethylstilbestrol — to healthy women, was regularly followed by a decrease of the Hp concentration in the blood, while a third estrogen compound — estriol — had no such effect. Estriol is also clinically different from other estrogen in that it mainly affects the uterine cervix and the vagina, while the endometrium is only to a slight extent influenced by this compound (Puck, Korte & Hübner 1957, Overbeek & De Visser 1958, Borglin 1959).

The decrease in circulating Hp, which was found when the two other estrogen compounds were given to healthy women, was not appreciably influenced by age nor by the functional state of the ovaries. During the first 5—7 days of treatment it was proportional to the total amount of the drug ingested.

Further administration resulted in a stabi ization of the Hp concentration on a lo level. After withdrawal of the drug the H concentration reached its original level wit in less than a week.

The decrease of the level found in health women in connection with ovulation (Ny man 1959) may thus be connected with th increase of circulating estrogens occurri during this period.

As to the mechanism of the action of th estrogens on the Hp values recorded, the are many possibilities. We have tried to an lyze the most obvious ones:

I) Estrogen are known to have a wat retaining effect resulting in hemodilutio with an apparent decrease in the serum pr tein concentration.

The electrophoretic studies reported und E show clearly that the percentual decrea in the Hp concentration exceeds by far th of any other serum protein fraction. Only small part of the depression found could th be attributed to hemodilution.

II) The methods actually available f quantitative Hp determination are all bas on the Hb binding properties of this protei A substance interacting with Hp or Hb cou decrease the affinity of the two componen to each other, with a resulting apparent d crease in the Hp concentration recorded. Th reports of Antoniades, Mc Arthur, Penn Ingersoll, Ulfelder & Oncley (1957) a Bischoff, Turner & Bryson (1958) sugg that estrogens are bound by some serum gl bulins of the alpha fraction not furth specified.

In our studies, addition of the estrog

pounds to serum *in vitro* did not in-nce the results recorded. On the other d Szego (1957) has claimed that *in vitro* ling of estrogens by serum proteins oc-s only in the presence of liver tissue. The sibility of a reaction between estrogens or e estrogen metabolites and Hp *in vivo*, a resulting decrease of its Hb binding acity, can not be excluded.

II) An increased breakdown of erythro-s with liberation of hemoglobin into the ulation has been shown to be followed decrease of circulating Hp (Laurell & nan 1957, Allison & Rees 1957, Nosslin Nyman 1958, Neale, Aber & Northam 8, Nyman, Gydell & Nosslin 1959).

Diethylstilbestrol has been reported to e some hemolytic effect *in vitro* (Ponder 8, Tateno & Kilbourne 1954). The oc-rence of some hemolysis *in vivo* is sug-ed by the studies of Feuchtinger (1940), izza, Notario & Ventura (1949) while ula & Leo (1956) did not find any nge in the amount of bile pigment ex-ed in feces and urine after administration large amounts of diethylstilbestrol to ans (30 mg a day for 10 days).

We found no change in the osmotic resi-ce of the red cells in subjects treated with ogens, but the increase of the COHb was statistically significant. This sug-s that there is an increase of the amount Hb catabolised, at least during the first k of the treatment. This conclusion is her confirmed by the fact that, in cases ted with estriol, in whom there was no ease of the serum Hp level, the COHb did not increase.

o judge from the number of circulating

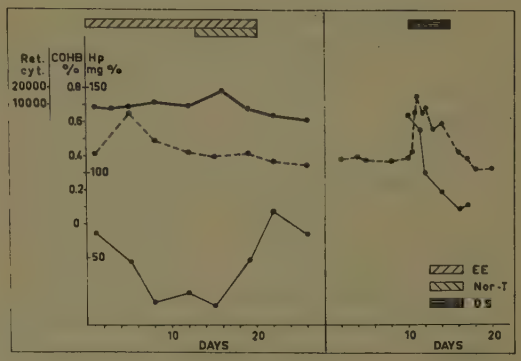


Fig. 6. Variations of the Hp level (mg per 100 ml serum) and COHb level (percentage) in 2 healthy women. To the left during and after 3 weeks of treatment with ethinylestradiol (group II). To the right before, during and after 5 days of treatment with diethylstilbestrol.

– – – – – COHb
——— Hp
——— reticulocytes
EE: ethinylestradiol
Nor-T: 17-α-methyl-19-nortestosterone
DS: diethylstilbestrol.

reticulocytes, however, there was no demonstrable response of the bone marrow even after administration of estrogens for 3 weeks (Fig. 6).

IV) Estrogens have been shown to stimulate the activity of the RES *in vivo* (Biozzi, Halpern, Bilbey, Stiffel, Benacerraf & Mouton 1957, Heller, Meier, Zucker & Mast 1958).

On the other hand patients with splenomegaly have been found to have lower haptoglobin levels in the blood than would be explained by the sometimes fairly slight increase in erythrocyte turnover found in these cases (Nyman 1959).

It is thus possible that there are some

connections between the increased RES activity due to estrogen stimulation and the decrease in Hp concentration found in our material.

V) A decrease of the Hp concentration has also been found in patients with acute or chronic liver cell failure (Jayle & Vallin 1952, Nyman 1959).

The compounds used are, however, not known to cause any liver cell damage and further the GPT level in the blood was unchanged during the whole observation period.

SUMMARY

The haptoglobin level of the blood has been found to decrease during administration of diethylstilbestrol and ethinylestradiol to healthy women of different ages. Estriol did not show this effect.

During the first week of treatment the decrease of the Hp level was a function of the total amount of estrogen given, while further treatment resulted in a stabilization of the Hp values on a low level.

This decrease was not due to hemodilution and did not seem connected with any effect of the estrogens on the liver cells or on blood coagulation factors.

At least in part the decrease seems to be connected with an increased breakdown of erythrocytes owing to the action of estrogens.

The well known effect of estrogens on the phagocytic activity of RES is possibly involved in this mechanism.

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THE SIGNIFICANCE OF THE EXTRAHEPATIC ELIMINATION OF ETHANOL IN DETERMINATION OF HEPATIC BLOOD FLOW BY MEANS OF ETHANOL

(A COMPARISON BETWEEN THE ETHANOL AND BROMSULFALEIN METHOD)

By J. A. LARSEN[1], N. TYGSTRUP, and K. WINKLER

From the Department of Surgery, The Finsen Institute, and the Finsen Laboratory, Medical Department B and Cardiological Laboratory of Medical Department B, Rigshospitalet, Copenhagen, Denmark

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Two methods for determination of the hepatic blood flow by means of ethanol without liver vein catheterisation have been suggested earlier (Larsen, 1958). These methods gave results which indicated a considerable extrahepatic elimination of ethanol, and therefore the findings had to be corrected for this amount. An indirect method for calculation of extrahepatic elimination of ethanol was described.

The present work was performed in order to assess the significance of extrahepatic elimination of ethanol when using these methods by comparing the values for hepatic blood flow obtained by the ethanol methods with those of the bromsulfalein method. Furthermore the hepatic - and thereby the extra hepatic - elimination of ethanol has been determined from the arterio-hepatic venous differences and the hepatic blood flow as measured by bromsulfalein.

[1] Present adress: Institute of Medical Physiology, University of Copenhagen, Juliane Mariesvej 28, Copenhagen, Denmark.

METHODS

The patients were examined in the morning b fore their first meal, they were recumbent duri the procedure and premedicated with 200 mg phenobarbitone.

Ethanol was administered intravenously, eith as a single injection, given in the course of minutes, or as an infusion from a calibrated i fusion pump, following a priming dose of 1500 m of ethanol.

For determination of hepatic blood flow patients received a continuous infusion of 2 to mg of bromsulfalein (Hynson, Westcott & Du ning) per minute, preceeded by a priming dose 100 to 150 mg.

Via an antecubital vein, a catheter was i troduced into a hepatic vein of the right lobe the liver. At least one hour was allowed f equilibration after start of the administration ethanol, then blood was drawn into heparini syringes from the catheter and simultaneously fr a polythene catheter in the femoral artery. T sampling was repeated every 15 minutes for hours. The blood concentration of ethanol w determined by the alcohol dehydrogenase meth described earlier (Larsen, 1959), and the broms falein was analyzed in plasma by Gaebler's meth (1945).

lations

e calculation of hepatic blood flow from peri- l ethanol concentration may be summarized as vs:

Single injection (Method I): The concentra- t which the elimination curve starts to flatten ken to represent the arterio-hepatic venous ence in the preceeding period. The total eli- ion of ethanol is calculated from the elimina- curve (Widmark, 1932) and the hepatic blood is calculated as total elimination divided by oncentration at flattening of the elimination .

Continuous infusion (Method II): A constant nt of ethanol is infused, smaller than that olized during the rectilinear part of the eli- ion curve after a single injection. After 60 es the peripheral concentration is constant. hepatic blood flow is calculated by dividing mount infused per minute by this concentra- which is taken to be identical with the arterio- ic venous difference.

The hepatic blood flow was determined by bromsulfalein according to Bradley, Ingelfinger, Bradley & Curry's method (1945).

In the present experiments hepatic blood flow was also calculated by substituting the actual arterio-hepatic venous differences for the concentration at flattening of the ethanol elimination curve.

MATERIAL

Eight patients without demonstrable hepatic or metabolic disorders were examined. Furthermore three patients suffering from cirrhosis of the liver were included, all at a rather advanced stage of the disease. Clinically their liver function was roughly reduced to the same extent.

RESULTS

The results of the experiments with a single injection of ethanol are summarized in Table I, and those with continuous in-

Table I. *Determinations of hepatic blood flow by ethanol (single injection) and by bromsulfalein, with calculation of the hepatic and extrahepatic elimination of ethanol.*

- t	Ethanol injected	Total elimination of ethanol	Ethanol				EHBF Bromsulfalein	EHBF (Bromsulfalein) in per cent of EHBF (Ethanol) direct Method	Hepatic elimination of ethanol	Extrahepatic elimination of ethanol
			Method I		Direct Method**					
			a	EHBF	a–h	EHBF				
	g	mg/min	mg/l	ml/min	mg/l	ml/min	ml/min	per cent	mg/min	mg/min
	10.92	78	55	1420	52	1500	1290	86	67.1	10.9
	10.92	90	50	1800	52	1730	1350	78	70.2	19.8
	15.66	103	45	2290	46	2240	1910	85	87.9	15.1
	11.74	69	–***	–	40	1730	990	57	39.6	29.4
	9.40	55	45	1220	25	2200	1590	72	39.8	15.2

Patients with cirrhosis of the liver.

**Method I is based on the peripheral curve alone, a is the concentration at the flattening of the elimi- ion curve, EHBF is the calculated hepatic blood flow. Direct method is based on the peripheral curve well as on the hepatic venous curve, a–h is the average arterio-hepatic venous difference during the tilinear part of the elimination curve.

***In case 4 the elimination curve was not followed long enough to show the deviation from the rectilinea rse.

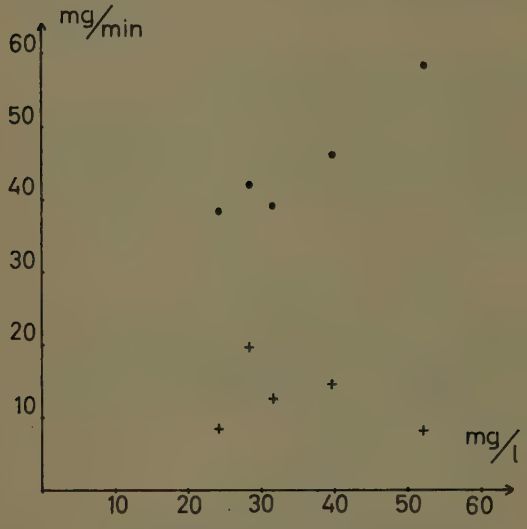

Fig. 1. The relation between ethanol concentration in peripheral blood and hepatic- and extrahepatic elimination.

. is the hepatic elimination.
+ is the extrahepatic elimination.

fusion in Table II. Both types of experiment show that under the conditions used, extraction of ethanol by the liver is practically complete in patients with normal liver fun tion. From this it follows that the figures f the hepatic blood flow by "indirect" (Met od I) and "direct" calculation are identic (Table I). In the three patients with ci rhosis of the liver, however, the extracti of ethanol was found to be incomplete.

The values for hepatic elimination of eth nol can be computed from the directly dete mined arterio-hepatic venous difference a the flow calculated by the bromsulfale method. As seen from Tables I and II, t hepatic elimination of ethanol in all cases lower than the total elimination, indicati the existence of an extrahepatic eliminatio In Fig. 1 the results from Table II are plot ed in a graph with the ethanol concentrati in the peripheral blood for the abscissa a the hepatic and extrahepatic elimination f the ordinate. It is seen that the hepatic e mination is roughly proportional to the eth nol concentration within the concentrati range examined. This proportionality is consequence of the extraction been practi

Table II. *Determination of hepatic and extrahepatic elimination of ethanol during continuous infusion of ethanol using liver blood flow estimated by bromsulfalein.*

Experiment No.	Ethanol infused (Total elimination)	Ethanol		EHBF Bromsulfalein	Hepatic elimination of ethanol	Extrahepatic elimination of ethanol
		a	a–h			
	mg/min	mg/l	mg/l	ml/min	mg/min	mg/min
6	61.5	28.3	28.3	1480	41.9	19.6
7	51.6	31.8	31.2	1250	39.0	12.6
8	44.4	24.2	22.9	1670	38.2	6.2
9	60.6	39.9	37.3	1230	45.9	14.7
10	66.2	52.2	49.6	1170	58.0	8.2
11*	70.3	84.5	50.4	1090	54.9	15.4

*Patient with cirrhosis of the liver.
a is the average peripheral concentration at level. a–h is the average arterio-hepatic venous difference.

complete in the normal liver at the con-
ation levels used.
ie difference between the total elimina-
and the hepatic elimination is the extra-
tic elimination. From Fig. 1 it is seen
the figures for the extrahepatic elimina-
calculated in this way scatter rather con-
ably without showing any definite de-
ence on the blood ethanol level. The
1 of the extrahepatic elimination in the
patients with normal liver function in
e I is 15.3 mg/min. as compared to 12.3
nin. in the patients with normal liver
ion in Table II.

DISCUSSION

previously demonstrated (Larsen
), the figure for the hepatic blood flow
lated according to Method II is depen-
on the blood ethanol level. As pointed
this may be due to an extrahepatic eli-
tion of ethanol, the amount of which
estimated to some twenty per cent of the
elimination under conditions of com-
saturation of the liver tissue with etha-
A calculation of the hepatic blood flow
Method II in the present investigation is
equently without any practical value.
ie rectilinear portion of the elimination
e for ethanol indicates a constant elimi-
n, and as the extrahepatic elimination of
nol is supposed to be constant down to
small concentrations, the figures for
tic blood flow obtained with Method I
ld be in constant relation to flow values
ned simultaneously by means of the
sulfalein method. The results are in
ement with the assumption of an extra-
tic elimination of ethanol amounting to about twenty per cent of the total elimination under conditions of complete saturation of the ethanol-removing mechanism in the liver (Table I, 3rd column from left).

By using a quite different principle for the evaluation of the extrahepatic elimination of ethanol one of us (Larsen 1959) found an average figure of 20.5 mg/min. The few determinations in each group do not allow us to state which figure for the extrahepatic elimination is the most correct.

The experiments presented in this paper further demonstrate that the extraction of ethanol in the normal liver is practically complete when the blood ethanol concentration is below a certain level, as in the case of galactose (Tygstrup & Winkler 1958). This level is indicated by the concentration at which the elimination curve after a single injection of ethanol begins to flatten. The high extraction percentage — in the infusion experiments it average 96 per cent — supports the view that practically all the blood passing the liver comes in contact with functioning liver cells and therefore functional intrahepatic shunts do not exist in cases with normal liver function. Whether the incomplete extraction seen in patients with cirrhosis of the liver is due to such shunts or to universally decreased cellular function cannot be established.

Our observations moreover confirm the existence of an extrahepatic elimination of ethanol which invalidates the use of ethanol for determination of the hepatic blood flow. Only when a reliable and fairly simple method for determination of the extrahepatic elimination is found can hepatic blood flow be determined with ethanol, and then without liver catheterisation.

The possibility can not be ruled out, however, that a correction of the total elimination by a constant factor for the extrahepatic elimination of ethanol per min/kg might suffice to allow an estimate of the hepatic blood flow from the elimination curves in peripheral blood.

The assumption made by Larsen (1959) that the extrahepatic elimination is independent of the blood ethanol concentration even at very low concentrations is to some extent supported by our observations but the material is too scanty to be decisive.

The existence of an extrahepatic elimination of ethanol does not exclude the usage of determination of the rate of ethanol elimination as a quantitative test for liver function, but it naturally decreases the sensivity of such a test. The fact that the extrahepatic elimination of ethanol must become relatively more important when the hepatic elimination is decreased may be one of the reasons why determinations of the elimination rate have not gained ground as a measure of liver function. As shown by Bernstein & Staub (1948) the elimination of ethanol is not significantly decreased until the function of the liver is seriously impaired.

It has been shown by Mendeloff (1954) that ethanol may increase the hepatic blood flow. However, in experiments with dogs, no such effect could be demonstrated (Smythe, Heinemann & Bradley 1953) and Castenfors, Hultman & Josephson (1960) did not find any effect of ethanol on liver blood flow in almost identical experiments in humans.

In the present experiments the hepatic blood flow was not determined before administration of ethanol, but great alterations caused by the amount of ethanol used are unlikely as the average blood flow value identical with that found earlier in this a other laboratories. However, this questi does not interfere with the conclusions dra from the present comparisons between sim taneous determinations of the hepatic blo flow by the ethanol and bromsulfale methods.

SUMMARY

The conditions necessary for calculati of the hepatic blood flow from the eliminati curve of ethanol in peripheral blood were i vestigated by hepatic venous catheterisati and determination of the hepatic blood fl by the bromsulfalein method. It was fou that the extraction of ethanol by the liv was almost complete (96 per cent) at l concentrations, except in patients wi reduced liver function due to cirrhosis of t liver.

The existence of extrahepatic eliminati of ethanol was moreover confirmed. Th extrahepatic elimination of ethanol inv lidates the use of ethanol elimination curv in the peripheral blood for determinations the hepatic blood flow, unless a simple c rection for the extrahepatic elimination c be made.

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AMYLOSE AS THE SUBSTRATE IN DETERMINATIONS OF AMYLASE

By K. JØRGENSEN and A. SVENDSEN

From The Department of Clinical Chemistry, Rigshospitalet, Copenhagen, and The Central Laboratory, County Hospital, Randers, Denmark

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In enzyme determinations, the use of a well-defined substrate is of obvious importance for a rational definition of enzyme units. For determination of amylase in blood and urine, this was aimed at by Street & Close (1956, 1958), who used purified amylose as a substrate. As their method seemed very satisfactory it was set up in our laboratories. Due to initial discrepancies between our results, despite only minor modifications in the procedure, some methodological studies were made. Our results, in part supplementing the papers of Street & Close, are given.

METHOD

The experiments were based on the following procedure, which is essentially as described by Street and Close (1958).

500 µl of phosphate buffer (20 mM, pH 7.0), 200 µl of amylose solution (0.1 per cent in 10 mM NaOH) and 200 µl of hydrochloric acid (10 mM) are mixed and prewarmed at 38.0° C (water thermostat). At time zero, 100 µl of sample are added (serum diluted 1:10, urine diluted 1:3, 1:9 *etc.* with physiological saline). After the incubation period (*e. g.* 10 min.) the test tube is removed from the thermostat, and the reaction is stopped by adding 8.50 ml of water. A blue color is developed by subsequent addition of 500 µl of iodine-iodide (10 mN I_2 in 20 mM KI). The absorbance is measur at 620 nm in a 10 mm cuvette against water.

The blind is mixed in the same way, but pr warming and incubation are omitted.

The spontaneous hydrolysis, which should negligible, is tested by replacing the sample wi physiological saline.

RESULTS

Amylose, and its blue color with iodine.

Amylose was prepared rather easily the procedure of Street & Close (1956), a our preparations yielded the same results a commercial product[1] which has been us for the studies herein described. Howeve the preparation of a substrate solution wi a preset amount of amylose is impossible practical work as the quantitative transfer the ethanol-moistened powder to the alkali solution is extremely difficult. The streng of the solution is probably better characte ized by the intensity of the blue color giv with iodine-iodide.

The blue complex with iodine-iodide w found to have an absorption curve similar

[1] Amylose from potato starch, British Dr Houses Ltd.

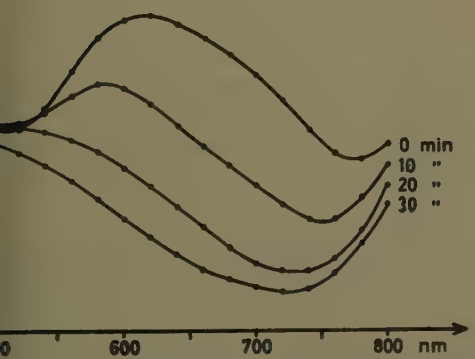
reported by Street & Close (1956). The absorption maximum at 620 nm was displaced slightly by addition of sample um or urine dilution). The extinction nds a little on the concentrations of ıe and iodide. In the present work the entrations were slightly higher (1.25 s) than given by Street & Close, which, ever, does not affect the conclusions vn.

he proportional relationship between entration of amylose and extinction of odine-complex has been confirmed.

hydrolysis, and the order of reaction.

he hydrolysis has been followed in seve- experiments. Fig. 1 shows the absorption tra of the blue color after hydrolysis for), 20 and 30 minutes, respectively. Dilut- erum was used as enzyme source.

rom the data in Fig. 1 hydrolysis curves be constructed, showing the decreasing rbance as a function of time at different elengths. Such curves are shown in



1. Absorption spectra of amylose-iodine color r various degrees of enzymatic hydrolysis.

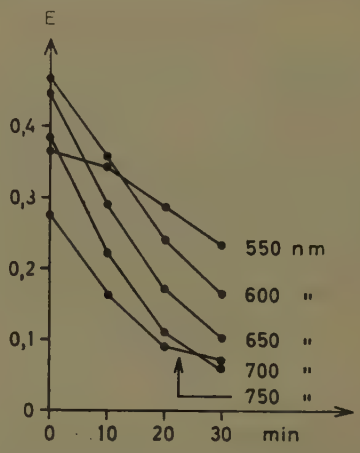


Fig. 2. Hydrolysis curves of the enzymatic hydrolyses of amylose, measured at various wavelength.

Fig. 2, from which it is seen that the function is rectilinear for the longest time when measured at about 620 nm *i. e.* the absorption maximum of the pure amylose-iodine color. When so measured the order of reaction apparently can be taken as zero, if the final absorbance is greater than half the initial value, *i. e.* above 0.200—0.250. This conclusion was confirmed by all similar experiments.

From the data in Fig. 2 some other relationships of interest can be derived. If the first 10 minutes' decrease of absorbance is taken as a measure of the enzyme activity, this will be found to vary with the wavelength employed as shown in Fig. 3, curve A. If the relative decrease is used instead, *i. e.* 10 minutes' decrease divided by initial absorbance, the dependence on wavelength will be represented by curve B. These results indicate that the kinetics of the hydrolysis is rather complicated.

In a series of experiments the initial re-

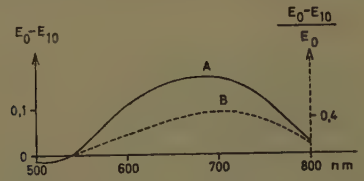


Fig. 3. The initial reaction velocity of enzymatic hydrolysis of amylose, as a function of the wavelength employed. Curves A and B show the reaction rates expressed as the decrease, and as the relative decrease, respectively, during the first ten minutes of hydrolysis.

action rate, measured as the fall of absorbance (620 nm) during the first ten minutes, was plotted against varied initial concentration of amylose, measured by initial absorbance. Some of these curves, obtained with different sera, are shown in Fig. 4. In this graph a horizontal line would indicate a zero order, and a straight line through the zero point would mean a first order reaction. Such experiments also illustrate that the kinetics cannot be described in a simple way.

As regards the enzyme, a proportionality was confirmed between the amount of enzyme and the reaction rate, measured as the decrease of absorbance at 620 nm during the first ten minutes, when the initial absorbance was about 0.500 and the final above 0.250.

CONCLUSION

For determination of amylase, the method of Street & Close (1956) is very convenient, especially on the ultra-micro scale (Close & Street, 1958). To obtain comparable results, however, the following points should be considered.

The initial amylose concentration must be fixed, and as complete dissolution of amylose powder is practically impossible, it should be characterized by the extinction of the iodine complex in the final dilution of the "stan dard" (Street & Close), *i. e.* in the abov mentioned "spontaneous hydrolysis blind" An absorbance of 0.500 (620 nm, 10 mm cuvette) is suitable with the concentration of I_2 and KI given above.

The wavelength should be fixed a 620 nm.

The extinction after incubation should b above 0.250, which secures a rectilinear de crease of absorbance within the incubatio period.

The catalytic effect should be charac terized by the initial reaction rate, measure as the decrease of absorbance per unit o time. The initial absorbance should be meas ured as a "test blind" (cf. "procedure" including sample, as this value may deviat significantly from "spontaneous hydrolysi blind".

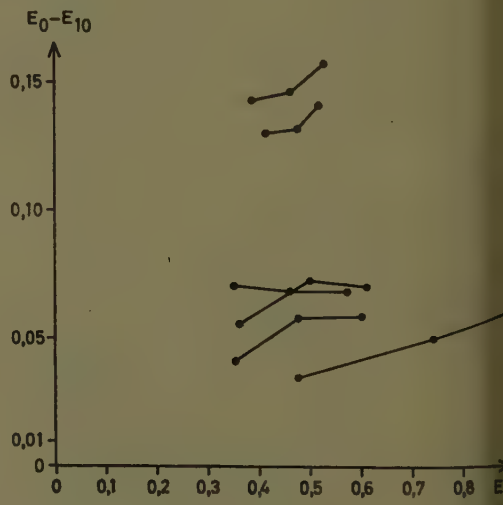


Fig. 4. Enzymatic hydrolysis of amylose. Th initial reaction rate, measured as 10 minutes' d crease of absorbance, as a function of the initi absorbance at 620 nm.

SUMMARY

Iethodolocigal studies on the amylase hod of Street and Close were carried out. ıe points of importance for the compara- y of the results are emphasized.

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EFFECT OF SEDATIVE DOSES OF NEMBUTAL ON THE RESPIRATORY METABOLISM

By E. HIRSJÄRVI and F.-E. KRUSIUS

From The Central Laboratory of The Kivelä Hospital, Helsinki, Finland

(Received for publication August 7, 1960)

The long-established drawback in all metabolic studies is the disturbing effect of muscular tension, which renders the results especially unreliable in neurotic patients. Attempts to overcome this effect have been made along two lines, *i.e.* to record the muscular twitches and to depress them. For the latter purpose the use of sedatives has been advocated. Rapport, Curtis & Simcox (1951) used intravenous injections of 100—500 mg Nembutal (Na-pentobarbital, Abbot) given at a slow rate, and measured the metabolic rate when sleep supervened. Later investigators used a smaller dosage, intended to induce a sedative effect without actually causing sleep. According to Hortling & Hiisi-Brummer (1957), the Nembutal metabolic rate (NMR) is in slightly better accordance with the clinical picture as well as with other tests of thyroid function, such as protein-bound iodine and serum cholesterol, than the BMR. In their opinion, however, the NMR still remains a method of limited reliability.

In the earlier studies the basal metabolism and somnolent metabolism have been measured with a spirometer which records only the oxygen consumption. The other parameters concerned in respiratory metabolism (CO_2,RQ) have not been measured. Nembutal, like other barbiturates, is, however known to have a depressant effect on t respiratory center, and, when administe ed in narcotic doses, to cause hypove tilation and an ensuing unsteady respir tory state with carbon dioxide retenti and a decrease of the respiratory (in co parison of the true metabolic) RQ (Rahn Otis 1949; Suskind & Rahn 1954). Ther fore it was considered necessary to investi ate whether Nembutal in sedative dos might have a similar effect, and to evalua its usefulness in clinical determinations metabolic rate in the light of the results o tained.

EXPERIMENTAL

Sixty-two patients were, 30 minutes after t usual determination of BMR, given injections 80—250 mg of Nembutal intravenously (usua about 1.7 mg per 1 kg of body weight), and t metabolic rate measured again without del On 13 subjects the test was repeated after 1- days, so altogether 75 Nembutal-metabolic r determinations were made. As a control 47 patie were submitted to two successive BMR measu ments, a rest of 30 minutes was allowed betwe the measurements. The test subjects were cho at random, from in and out patients of the Kiv hospital. The average BMR of the Nembutal gr was, however, somewhat higher than that in

l group (+ 33.9 ± 2.25 and + 24.0 ± 3.02
tively). Approximately one half of the pat-
fell asleep before or during the NMR deter-
on. The effect on metabolism as well as on
vas stronger in the sleeping subjects. There
however, no clear correlation between the
of Nembutal and the sleep, nor between the
nd the effect on metabolism and RQ.
e measurements were carried out with a Hart-
-Braun katharometric apparatus. The factors
red were the minute volume of ventilation,
atory-expiratory oxygen difference (oxygen
) and expiratory carbon dioxide content (car-
ioxide gain), all measured as the averages of
nutes (the inspiratory carbon dioxide, being
ificant, is disregarded in the method). The
atus was calibrated for oxygen and carbon
e with the micro-Scholander gasometric
atus and the gas meter with known amounts
. The oxygen consumption and RQ were cal-
d on the basis of these factors. The BMR was
ssed as percentages of standard values, derived
generally used nomograms.

RESULTS AND CONCLUSIONS

e results are presented in Tables I—III.
ne Nembutal group the initial BMR
varied from —7 to + 76 per cent,
ging + 33.9 per cent. The values after
butal administration (NMR) varied
een —2 and + 68 per cent, except in
case, where the metabolism dropped
an initial BMR value of + 18 per cent
-26 per cent after Nembutal, the same
nts values on the next day were BMR
) per cent and NMR + 7 per cent. The
tion of Nembutal, in most cases, caused
rease in the metabolism, which averaged
± *1.05* (Table I), being of the same
itude as reported by Hortling & Hiisi-
nmer (1957). The factor responsible for
eduction in metabolism was the volume

T a b l e I. *Effect of intravenous administration of Nembutal (Na-pentobarbital) on the gaseous exchange*

Oxygen deficit per cent			Carbon dioxide gain per cent			Volume of ventilation l/min.			Respiratory quotient			Metabolic rate, per cent difference from standard		
Control	Nembu-tal	Differ-ence	Control	Nembu-tal	Differ-ence	Control	Nembu-tal	Differ-ence	Control	Nembu-tal	Differ-ence	Control	Nembu-tal	Differ ence
4.46 ±0.078 n=75	4.59 ±0.072	+0.132 ±0.040	3.55 ±0.054	3.45 ±0.046	—0.074 ±0.034	6.45 ±0.154	5.98 ±0.153	—0.469 ±0.076	0.800 ±0.0050	0.759 ±0.0047	—0.0407 ±0.0053	+33.9 ±2.25	+26.1 ±2.30	—7.65 ±1.05

Table II. *Comparison of the gaseous exchange during two successive BMR measurements*

Oxygen deficit per cent			Carbon dioxide gain per cent			Volume of ventilation l/min.			Respiratory quotient			Metabolic rate, per cent difference from standard		
1. st BMR	2. nd BMR	Difference	1. st BMR	2. nd BMR	Difference	1. st BMR	2. nd BMR	Difference	1. st BMR	2. nd BMR	Difference	1. st BMR	2. nd BMR	Difference
4.56 ±0.080 n=47	4.48 ±0.079	—0.076 ±0.050	3.58 ±0.050	3.56 ±0.060	—0.018 ±0.030	5.66 ±0.166	5.67 ±0.175	+0.011 ±0.077	0.788 ±0.0066	0.796 ±0.0058	+0.0052 ±0.0055	+24.0 ±3.02	+22.7 ±3.19	—1.25 ±0.76

of ventilation, which showed a marked d crease in the majority of cases whereas th oxygen deficit remained unchanged or i creased. Thus, the reduction in ventilati exceeds the reduction in oxygen co sumption, without evidence of oxygen d ficiency. The expiratory-inspiratory carbo dioxide difference, on the other hand, show ed a consistent, though not very marke decrease, indicating decreased carbon dioxid output in comparison with the oxygen co sumption, accordingly the RQ was lowere (average difference before and after Nem butal —0.0407 ± 0.0053). The differen between the control and Nembutal valu was statistically significant as regards th oxygen deficit, ventilation volume, and RQ and statistically probable as regards the ca bon dioxide gain.

In the control group there were no marke changes measured or calculated in any of th factors (Table II). The initial BMR valu in this group varied between —37 and + 6 per cent, the average value being + 24. ± 3.19 per cent. The BMR values in th repeated test varied between —25 and + 6 per cent, averaging + 22.7 per cent. Chang in the oxygen deficit, carbon dioxide ga and ventilation were insignificant, as cou be expected.

These results indicate that Nembutal, eve in relatively small doses, causes hypovent lation and a consequent unsteady respirator state with carbon dioxide retention, at lea during the period of metabolic rate dete mination. Thus, determination of NM actually means determination of metabolis in an unsteady state, which hardly can considered as physiologically sound. If, how ever, it could be shown that the NMR ga

e consistent values from day to day in same patient than the BMR, as claimed Rapport, Curtis & Simcox (1951), *i. e.* Nembutal would depress the metabolism steady standard value, less susceptille to ıce deviations than the BMR, the clinical of NMR could be recommended, though. standard values would have to be ked out for its correct intepretation.

o test this, 13 patients were submitted wo successive BMR and NMR determi- ons. The test was performed as in the ve-mentioned Nembutal group (in which e results are included) *i.e.*, after the ini- BMR measurement the patient was given mg Nembutal/kg body weight intrave- sly, and the measurement repeated. A nd test was performed in the same way r 1—4 days. In addition one patient was nitted to two NMR measurements on erent days, without a foregoing BMR surement. The results are given in le III, which presents a comparison veen the two successive BMR and the successive NMR determinations. It is that in the NMR as well as in the BMR ıp, the second determination gave slightly er values. The difference, however, is less ked in the NMR group. This is largely to a considerable increase of metabolism wo patients complaining of sickness dur- the second NMR determination. As re- ls the consistency of the individual pati- s values in the two groups, the scatter arkedly greater in the NMR group, the n error of the mean difference being more 2.5-fold that in the BMR group. The tly greater mean error in the mean RQ erence in the NMR group is in accord- with this. Though the greater inconsi-

Table III. *Comparison of NBR and BMR, measured on two different days*

Nembutal						Control					
Metabolic rate, per cent Difference from standard			Respiratory quotient			Metabolic rate, per cent Difference from standard			Respiratory quotient		
1st exp.	2nd exp.	Difference	1st exp.	2nd exp.	Difference	1st exp.	2nd exp.	Difference	1st exp.	2nd exp.	Difference
+25.9 ± 5.1 n=14	+23.9 ± 4.5	—1.86 ±2.82	0.749 ±0.010	0.756 ±0.009	+0.0075 ±0.013	+33.6 ±5.6 n=13	+28.0 ±5.2	—5.2 ±1.08	0.794 ±0.110	0.803 ±0.007	+0.0080 ±0.012

stency of the NMR determinations is also mostly due to the above-mentioned two cases which showed a marked increase in the second NMR determination, it is not possible to forecast which patients will react to Nembutal with sickness or other discom-

fort, rendering the values unreliable. On the other hand, if these two cases are excluded, the constancy of the two determinations still is slightly better in the BMR group (average difference between the two BMR determinations —5.6 ± 1.08, and between the two NMR determinations —5.7 ± 1.63).

Thus, the present material indicates that the NMR, besides being unphysiologic, is no more reliable in the measurement of the true metabolic rate than the usual BMR. Therefore, carefully controlled BMR measurements, preferably on different days, should be recommended instead of NMR.

SUMMARY

In order to test the effect of sedative doses of Nembutal on the gaseous exchange, determinations of ventilation, oxygen consumption, carbon dioxide output, respiratory quotient and metabolic rate were carried out before and after intravenous administration of 80—250 mg of Nembutal. Nembutal had a moderately depressing effect on the metabolic rate, and a marked depressing effect on the respiratory quotient, indicating carbon dioxide retention due to hyperventilation.

Comparision of repeated BMR and NMR determinations indicate that the NMR values on different days are slightly less constant than the BMR values.

ACKNOWLEDGEMENTS

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DETERMINATION OF ARGINASE IN SERUM

By O. M. FORSELL and I. P. PALVA

From The Central Hospital of Northern Carelia, Joensuu, Finland

(Received for publication August 7, 1960)

ginase was described by Kossel & as early as 1904. Several workers have found it in various tissues, in greatest ities in the liver. Its significance was ished by the cycle by which amino en produces urea during the catabolism ino acids (Krebs & Henseleit 1932). ginase hydrolyses arginine into ornith- d urea with a pH optimum of 0.9—9.8. tivity has been measured by determin- ne urea either enzymatically in Con- chamber or chemically with diacetyl- xime. Arginase has been determined cells and whole blood, but the method ot sensitive enough to demonstrate it in (Clak & Beck 1949, Frideman & r 1955, Reynolds, Folette & Walen- 957). Manning & Grisolia (1957) were o determined arginase activity from the nium sulphate precipitate of serum pro- The present authors tried to increase nsitivity of the arginase determination mit reliable measurement directly from .

METHOD

0.1 ml of serum were added 1.25 ml of distill- er, 0.05 ml of 10 per cent $MnCl_2$, 0.1 ml of $CoCl_2$ and 0.5 ml of 0.2 M arginine mono- hydrochloride (BDH). The pH was adjusted to 9.5 and the solution was incubated for 6 hours at 38° C. To the solution were added 1 ml of 10 per cent trichloracetic acid and 2 ml of 0.1N HCl, and it was centrifuged for 15 minutes. The urea in 1 ml of the filtrate was determined by the diacetyl-monoxime method by heating at 2 atm pressure for 15 minutes in a domestic pressure cooker (Forsell & Palva 1960). In the control test, performed simultaneously, arginine was added only after incubation. This gave the amount of urea present in the reaction mixture without arginine hydrolysis. This was deducted from the result of the principal test.

The arginase unit selected by the authors was the activity required to hydrolyse one micromol of arginine in an hour at an arginine concentration of 0.05 M.

All tests and control tests in serum were done in duplicate for each series, together with a blind test without serum. Samples which were even slightly hemolysed were rejected because of the high arginase activity of the red cells.

To exclude the presence of inhibitors, the test tubes and pipettes were rinsed with distilled water treated with ion exchanger, and dried with acetone in an air stream.

To check the reability of the arginase determination method, the authors performed determinations with purified arginase (Fluka). Two determinations of each concentration were done and the mean taken as the result. Fig. 1 shows that the activities obtained were directly proportional to the weighed total of enzyme preparation used in the test. When the

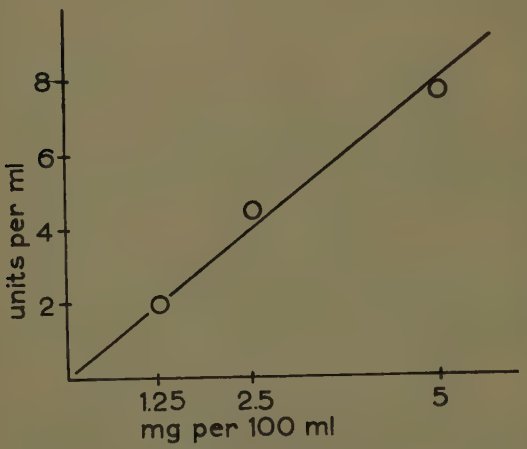

Fig. 1. The correlation of measured arginase activity with the weighed mean of purified enzyme preparation.

enzyme was added to normal serum, a mean recovery of 85 % per cent was obtained in 20 routine determinations.

Normal values

The authors determined arginase activity of the sera from their laboratory staff and blood donors of the Red Cross Blood Service in 20 young adults. The results are listed in Table I and varied from 0 to 2.8 units, mean 1.3, standard deviation 0.85 units and mean ± 2 S.D. = 0—3.0 units per ml.

DISCUSSION

The greatest drawback of the arginase determination methods available so far has been the small amount or urea. It has most commonly been determined enzymatically in Conway's chamber or chemically either diacetylmonoxime or isonitrosopropiophenon. The former method is rather elaborate and not sufficiently sensitive. The latter are sensitive, but the color formation does not obey Beer's law, especially in small concentrations. An additional drawback is that the pigments disintegrate readily when exposed to light. The authors therefore perform the urea determination with diacetylmonoxime at 2 atm pressure. With this method the extinction of the condensation product was directly proportional to the urea concentration in the range 0.80 mg of urea nitrogen per 100 ml of the sample. An additional advantage was the shortening of the reaction time from 60 to 15 minutes and the light stability of the pigment obtained.

To obtain a greater urea concentration for determination Manning & Grisolia (1955) incubated ammonium sulphate precipitate of serum with arginine. It is not known definitely whether the enzyme is unscathed in the precipitation and whether it is possible to measure with fair accuracy a quantity of precipitate corresponding to the volume used of the serum. The present authors tried to increase the quantity of urea to be measured and thus to detect even small variations.

Table I. *Serum arginase activities in 20 healthy adults.*

Person	Arginase units per ml
V. U.	0.0
M. P.	0.0
R. P.	0.28
E. T.	0.30
I. V.	0.56
T. P.	0.60
I. P.	0.80
O. T.	0.90
K. N.	1.1
O. F.	1.2
M. T.	1.2
A. S.	1.4
E. M.	1.7
H. N.	1.7
S. R.	1.7
K. P.	2.0
E. K.	2.1
M. T.	2.4
M. S.	2.8
K. S.	2.8

amount of arginase by lengthening the bation period to 6 hours instead of the monly used short time of 15—30 min-. The activators were manganese and lt. To exclude the presence of inhibitors, pulous precautions were taken in clean-the glassware employed.

SUMMARY

n arginase determination method suf-ntly sensitive to demonstrate enzyme ity in normal serum was achieved. The obtained in the hydrolysis of arginine determined with diacetylmonoxime by ing at 2 atm. pressure for 15 minutes. incubation period of the hydrolysis was thhened to 6 hours. The normal values ined for the sera of 20 healthy adults 0—3.0 units, mean 1.3 units per ml.

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THE GLYCOGEN CONTENT OF LEUKOCYTES FROM DIABETIC AND NON-DIABETIC SUBJECTS [1]

By V. ESMANN

From The Second University Clinic of Internal Medicine, Kommunehospitalet, Aarhus, Denmark

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The glycogen content of leukocytes from 15 poorly controlled, insulin-treated diabetics was reported by Valentine, Folette & Lawrence (1953) not to differ from a control group, the averages being 78 mg and 75 mg glycogen per 10^{10} myeloid leukocytes respectively.

During experiments on the carbohydrate metabolism of leukocytes incubated *in vitro*, it was noted that pre-incubation values of glycogen were rather low in leukocytes from some diabetic patients. The problem of the glycogen content of leukocytes immediately after isolation in patients with uncontrolled diabetes was therefore re-investigated.

METHODS

Isolation of leukocytes. — Venous blood was collected through a siliconed cannula into two precooled, siliconed 50 ml centrifuge tubes containing dextran[2] and EDTA in final concentrations of 1 per cent and 0.1 per cent, respectively. The tubes were immediately placed in an ice-bath and th erythrocytes allowed to settle. After 30 minutes th leukocyte-rich plasma was drawn off and centri fugated at 200 × g for 5 minutes. The leukocyte in each tube were washed once with 50 ml ice cold saline and centrifugated again for 2 minute Finally, the leukocytes were suspended in cold iso tonic saline, and 1 ml aliquots immediately draw for glycogen determination.

After withdrawal of the blood, the temperatu of the supernatant plasma fell to 7° C within minutes and remained there except during centri fugation, when it rose to 18° C. The total prepara tion time was 45 minutes.

The final suspension contained 30—60 per cen of the avaiable leukocytes, no thrombocytes an 2-3-(6) erythrocytes per leukocyte. The conta mination is negligible, as erythrocytes contain n glycogen (Wagner 1946). Leukocytes were counte in quadruplicate. The differential count was mad on 200 cells.

Glycogen determination. 1 ml leukocyte su pension was added to 1 ml 30 per cent (w/v) KO and the mixture was heated for 45 minutes in boiling water-bath. The method was otherwise described by Good, Kramer & Somogyi 1933 except that the glycogen precipitate was washe according to Walaas & Walaas (1950). Gluco was determined by the method of Somogyi (1952 and expressed in terms of glycogen by multiplyi by 0.90.

Glycogen was determined in triplicate and t result expressed in mg glycogen per 10^{10} myelo leukocytes.

[1] This investigation was supported by grants from the Danish State Research Foundation and Rheinhold W. Jorck and Wife's Foundation.

[2] Dextran, fraction TDR 205-II-B-I, MW: 310.000, intr. visc.: 0.40, was kindly supplied by A/S "Pharmacia", Sweden.

Table I. *Diabetic state at the time of investigation.*

	No. of subjects	Fasting Blood Sugar		Glucosuria			Ketonuria		Insulin Requirement	
		100–199 mg/100ml	≧200 mg/100 ml	0–0.9 per cent	1–1.9 per cent	≧2 per cent	Present	Absent	≧40 i. u.	None
ntrolled	14	3	11	0	3	11	8	7		
olled	10	10	0	9	1	0	0	10	12	2

MATERIAL

e non-diabetic series consisted of 25 subjects. were medical students, and the remainder ts with various neurological, rheumatoid, in- and pulmonary diseases. None had hema- cal disorders, febrile reactions or were under ıent with cortisone. The patients were classi- ıs non-diabetic after repeated negative tests inary sugar. Oral glucose-tolerance tests were rmed in six of the medical students.

e diabetic series consisted of 14 patients; in of these, the diabetes was of recent onset, the duration was less than 5 years in three ıore than 15 years in three. Insulin had never given in 11 of the patients, and was withheld 2 and 4 days, respectively, in the remaining Ten patients were re-investigated after re- on of the diabetes. The diabetic state before fter regulation appears from Table I, and e distribution of the two series from Table II. was a slight difference in the age distri- .

patients were fasting and had a complete rest for half an hour before blood samples were rawn.

RESULTS

e influence of the experimental condi- on the glycogen content was investigat- various ways.

Table III shows the results of experiments with parallel determinations of glycogen on leukocytes from blood sedimented in ice-bath and at room temperature (approximately 23° C). It was found that the average glycogen content was 8.7 per cent higher under the latter circumstances.

Table IV shows the results when the sedimentation time was varied. Prolonging the sedimentation at 4° C from 30 to 60 minutes caused a decrease in glycogen content in all but one experiment.

Preliminary washing of the leukocytes is necessary to remove the dextran, which would otherwise be determined as glycogen. In some experiments, patients with a very high spontaneous sedimentation rate were selected and the blood allowed to sediment at room temperature with and without added dextran (Table V). The glycogen values were identical in the two conditions, showing that dextran was efficiently eliminated by the washing. This result was confirmed in experiments with repeated washings of the leukocytes.

Table II. *Age distribution of diabetic and non-diabetic series.*

	No. of subjects	10–29 yrs.	30–49 yrs.	≧ 50 yrs.
Diabetics.....	25	28 per cent	32 per cent	40 per cent
Non-diabetics.....	14	43 » »	14 » »	43 » »

Table III. *Influence of temperature during sedimentation on glycogen-content of leukocytes+)*

Exp.	Sedimentation tp.		Difference per cent.
	Ice-bath mg	room-tp. mg	
1	178	183	+2.8
2	134	154	+14.9
3	76	82	+7.9
4	97	106	+9.3

Leukocytes were isolated as described under "Methods", except for the variation in temperature during sedimentation. Mean difference: +8.7 per cent, S.E.: 2.5, $t = 3.48$, $t_{0.05} = 3.18$.

+) mg glycogen per 10^{10} myeloid leukocytes.

A comparison of the glycogen content of leukocytes from diabetic patients before and after regulation of the diabetic state is subject to error, if any significant day-to-day variation in glycogen content occurs. In five non-diabetic and two diabetic patients glycogen was therefore determined on two consecutive days during which the patien apparently lived under the same conditio (Table VI). Experiment Nos. 6 and 7 sho ed fairly large percentage differences, but i these two experiments the absolute differe ces were small.

Figure 1 illustrates the results of glycoge determinations in 25 non-diabetic subjec and 14 diabetics with uncontrolled diabete The values obtained averaged 148 mg an 111 mg glycogen per 10^{10} myeloid leuk cytes, respectively, the differences being st tistically significant at the 2 per cent level.

The small difference in the age distributio

Table IV. *Influence of sedimentation time on glycogen content of leukocytes+)*

Exp.	Sedimentation time		Difference per cent.
	30 min. mg	60 min. mg	
1	170	161	— 5.3
2	156	124	—20.5
3	125	117	— 6.4
4++	173	195	+12.7
5	107	95	—11.2
6	141	124	—12.1

Leukocytes were isolated as described under "Methods", except for the variation in sedimentation time. Mean difference: —11.1 per cent, S.E.: 2.7, $t = 4.12$, $t_{0,02} = 3.75$.

+) mg glycogen per 10^{10} myeloid leukocytes.

++) Excluded in calculation of the mean.

Table V. *Influence of dextran on glycoge determination+)*

Exp.	Dextran sediment. mg	Spontaneous sediment. mg	Difference per cent.
1	134	138	—2.9
2	133	128	+3.9
3	129	128	+0.8
4	116	111	+4.5

To blood from patients with a high sedimentatio rate were added 1/5 volume dextran and 1/5 volum saline, respectively. Room temperature. Otherwis the isolation was performed as described unde "Methods". Mean difference: +1.6 per cen S.E.: 1.7.

+) mg glycogen per 10^{10} myeloid leukocytes.

of the two series did not exert any influenc on this result, as the average glycogen co tent of the non-diabetic subjects in ag groups 10-29, 30-49 and $\geqq 50$ years ar almost identical *viz.* 144, 157 and 143 m glycogen, respectively.

The subjects were classified as non-dia betic on the basis of aglucosuria. The mea

en content of the leukocytes from six
its, in whom aglucosuric diabetes was
out by oral glucose-tolerance tests,
ged 151 mg (S.E. 12 mg) which is
tent with the overall average of 148 mg.
the three patients with long-term dia-
the glycogen values were 67, 114 and
g. Lower as well as higher values were
ed among the patients with diabetes
rt duration. Nor could any difference
nonstrated between the ketotic and the
etotic diabetics, the average glycogen
t being 116 mg and 105 mg, respec-

ure 1 also shows the results of gly-
determinations on leukocytes from 10
diabetic patients after regulation of the
ic state. After treatment, the average
en content of the leukocytes from these
tients increased from 118 mg to 156
e increase being statistically significant
2 per cent.

le VI. *Day-to-day variation in gly-
gen content of human leukocytes*+)

1st day mg	2nd day mg	Difference per cent.
134	176	+31.3
269	244	— 9.3
79	77	— 2.5
99	107	+ 8.1
113	111	— 1.8
39	46	+18.0
67	50	—25.4

ycogen determination was performed on two
tive days on leukocytes from fasting, rest-
ients. Exps. 1—5: normal patients; Exps.
uncontrolled diabetic patients. Leukocytes
olated as described under "Methods". Mean
ce: +2.4 per cent, S.E.: 6.5.

glycogen per 10^{10} myeloid leukocytes.

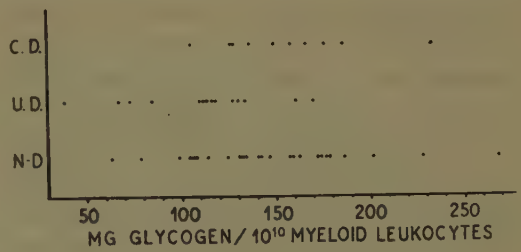

Fig. 1. *Glycogen content of human leukocytes.* Glycogen was determined on leukocytes from fasting non-diabetic and uncontrolled diabetic subjects after 30 minutes' complete rest in bed. The glycogen determination was repeated on 10 of the diabetic subjects after treatment. The glycogen content in mg per 10^{10} myeloid leukocytes averaged (± S.E.):

ND: Non-diabetics: 148 mg ± 9 mg
UD: Uncontrolled diabetics: 111 mg ± 10 mg
$t = 2.61$, $t_{0.02} = 2.46$

CD: Controlled diabetics: 156 mg ± 12 mg
$t = 3.02$, $t_{0.01} = 2.82$

Treated as paired observations the difference between groups CD and UD holds at the 2 per cent level, the mean of group UD in this case being 118 mg ± 10 mg.

DISCUSSION

The values obtained in glycogen determinations on leukocytes depend on the experimental conditions.

The experiments with different sedimentation times showed a fall in the glycogen content of approximately 10 per cent when sedimentation was prolonged from 30 to 60 minutes at 4° C, which suggests the possibility that a certain decrease also takes place during the first 30 minutes of sedimentation.

The glycogen content of leukocytes after sedimentation at room temperature was found to be approximately 10 per cent higher than that of leukocytes sedimented at 4° C, as was done routinely in the experiments reported here. Some glycogen synthesis at room temperature is possible, as appreciable glycogen synthesis occurs when leukocytes are incubated at 37° C *in vitro* (unpublished experiments).

Under the conditions described, the average glycogen content of leukocytes from non-diabetic subjects is appreciably higher than the 42 mg and 75 mg reported by Wagner (1946) and Valentine *et al.* (1953). In Wagner's series, the leukocytes were isolated by prolonged and fast centrifugation, which is known to damage the cells. This, together with a somewhat longer isolation time, is the most probable explanation of the lower values observed. The discrepancy with the Valentine *et al.* series is difficult to explain, but is probably also due to different experimental condotions. Valentine *et al.* used the fibrinogen method for the separation of leukocytes and the Anthron method for the glycogen determination.

As the wet weight of 10^{10} leukocytes is approximately 12 g (Bird, Clements & Becker 1951), the glycogen content represents 1—2 per cent of the wet weight of the leukocytes from fasting, non-diabetic subjects. This is of the same order of magnitude as the glycogen content in biopsy specimens from muscle and liver from fasting subjects (Hildes, Sherlock & Walshe 1949).

The demonstration of a lower glycogen content of leukocytes from diabetic patients is in accordance with the observation that, *in vitro,* the glycogen synthesis of diabetic leukocytes is decreased (unpublished expe ments).

The sensitivity of diabetic leukocytes insulin *in vivo* is apparent from the rever of the glycogen content to normal valu after treatment, and is paralleled by a high ability of leukocytes from controlled th from uncontrolled diabetics to synthesi glycogen *in vitro* (unpublished expe ments).

The effect of insulin treatment may e plain that Valentine *et al.* obtained norm glycogen values in leukocytes from insul treated diabetic subjects.

SUMMARY

The glycogen content of leukocytes fr diabetic and non-diabetic subjects was det mined on cells obtained by sedimentation the blood with dextran.

The influence of the experimental con tion was investigated; it was found that highly standardised technique is necessa to obtain reproducible results.

The glycogen content of leukocytes fr 25 non-diabetic fasting and resting subje averaged 148 mg (S.E. 9 mg) per 10^{10} my loid leukocytes.

The leukocytes of 14 diabetic patie without insulin and in a poorly controll diabetic state showed a significantly low glycogen content (111 mg (S.E. 10 mg per 10^{10} myeloid leukocytes, which return to normal after treatment.

ACKNOWLEDGEMENT

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ASSAY OF ANTIHEMOPHILIC A, B AND C FACTORS BY ONE-STAGE CEPHALIN SYSTEMS

By O. EGEBERG

From The Institute for Thrombosis Research, University Hospital, Rikshospitalet, Oslo, Norway

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In 1953, Langdell, Wagner & Brinkhous introduced a one-stage assay of coagulation factor VIII (antihemophilic A factor = AHA) based on the "partial thromboplastin" or cephalin time. The method was later used also for other antihemophile factors. The same year, Soulier & Larrieu published a recalcification time system for the assay of factor VIII and factor IX (antihemophilic B factor = AHB). This system was later modified to a cephalin system, and was also used to measure other intrinsic factors (Larrieu & Weilland 1957). Different modifications of these methods have been proposed (Geiger, Duckert & Koller 1955, Bounameaux 1957, Stapp 1958, Waaler 1959, a).

The one-stage cephalin method for the assay of intrinsic factors is simple and rapid. It is probably as sensitive and accurate as the more complicated systems based on the prothrombin consumption test (Graham, Collins, Godwin & Brinkhous 1951, Wolf 1956) or on the thromboplastin generation test (Biggs, Eveling & Richards 1955, Biggs 1957, Pitney 1956, Pool & Robinson 1959). However, the usual modifications of the cephalin assay system present still important problems as to reproducibility and specificity. In most systems the standard curve has a very modest slope, and slight differences in contact during the test, or in the stability of the substrate plasma, may highly influence the results.

Following a study of the sources of errors in a one-stage cephalin assay of antihemophilic C factor (PTA = AHC), a procedure for the assay of antihemophilic factors with cephalin systems has been worked out. The method is presented in this paper, together with some data on its accuracy, and on the measured levels of the factors in normal persons and in hemophiliacs.

MATERIALS AND METHODS

Materials.

Buffer. A modified veronal buffer according to Owren (1947) was used (pH 7.35, ionic strength 0.154).

Calcium. Solutions of calcium chloride were made by diluting a 1 M aqueous stock solution in distilled water. In clotting tests involving calcium optimal concentration was used; in the assay systems for antihemophilic factors this was regularly 30 mM.

Cephalin suspension. A crude suspension of the acetone-insoluble, ether-soluble fraction of human

was prepared according to the method of Rapaport & Owren (1955). This stock sus- was kept frozen at —20° C. It was diluted mal concentration in Owren's buffer, usually and stored at —20° C in stoppered acid- glass tubes. Thawing was done at 37° C, ie suspension was shaken vigorously be- e.

ated normal plasma. The plasmas were pre- by silicone technique ("silicone" plasmas). incture was done with a siliconized needle, e first 2—3 ml of blood were discarded. lood was then collected directly in chilled zed glass tubes containing 1/10 volume of per cent sodium citrate dihydrate, immedi- mixed with the citrate and cooled in ice Platelet-poor plasma was prepared by centri- n at 2,500 rpm. (max. *g* ca. 1,700) for 30 t 4° C, and the plasma was transferred to siliconized glass tubes with a siliconized The plasma was then rapidly frozen in uantities in stoppered siliconized glass tubes ored at —20° C. Frozen plasma was thawed C, and kept in ice bath before use.

ated deficiency plasma. Plasma deficient in or AHB was prepared from patients with hemophilia A and B. Plasma deficient in V (proaccelerin) was prepared from a pat- th bleeding tendency caused by a congenital ncy of this factor. Plasma deficient in AHC epared from two persons with severe defi- who, however, had no history of hemorr- liathesis. The patients were bled in a fasting The plasmas were prepared with the same ue as described for citrated "silicone" nor- asma. In some investigations the plasma was uged again at 30,000 rpm. (max. *g* ca. 81,000) min. at 4° C, and the middle layer in the ge tube was collected using siliconized . The deficiency plasma samples were either ice bath for immediate use, or rapidly frozen ored at —20° C. Thawing was done as for plasma.

tion fluids. When dilutions of plasma were in the assays, the plasma was first diluted in dilution fluid I to 10 times the final concentration, then the final dilution 1/10 was done in dilution fluid II. Dilution fluid I was made by mixing 100 ml of 3.13 g per cent sodium citrate dihydrate with 500 ml of saline. This fluid has approximately the same ionic strength and citrate concentration as citrated plasma from blood with a normal hematocrit. Dilution fluid II was made by mixing 200 ml of Owren's buffer, 200 ml of 25.66 mM sodium citrate dihydrate, and 600 ml of saline. This fluid has a pH of 7.35, an ionic strength of 154, and a suitable low concentration of citrate.

Siliconizing of equipment. Glassware, including the pipettes used for plasma and plasma dilutions, was siliconized with a 2 per mill aqueous solution of Dow Corning Z 4141 from Dow Corning Corporation, Midland, Michigan, U. S. *Needles for venepunctures* were coated with Monocote from Armour Laboratories, Hampden Park, Eastbourne, England.

Test tubes. New faultless unsiliconized glass tubes ("Elka" from Karl Hecht, Sondheim, Germany) were used. The tubes had an internal diameter of 8 mm and a length of about 60 mm; they were used only once. In some assays specially cleaned tubes were used. These tubes were soaked in chromosulfuric acid for 24 hours, rinsed in tap water, soaked in 10 per cent potassium hydroxide solution for 24 hours, rinsed in tap water, and finally rinsed in distilled water (Waaler 1959, b).

Methods.

Cephalin times were determined in the following way: 0.2 ml cephalin suspension of optimal concentration + 0.2 ml dilution fluid II + 0.2 ml undiluted test plasma were pipetted into the test tubes. The tubes were inverted 3 times and immediately incubated in water bath at 37° C. After incubation for 6 min., 0.2 ml of 30 mM $CaCl_2$, prewarmed to 37° C, was added, starting the stop watch at this moment. The tubes were immediately inverted 3 times; after 45 sec. incubation, they were tilted every 10 sec. The end point was determined under side illumination of the test tube against a black back-ground. The watch was stopped at the moment when coagulation spread generally in the

Table I. *The influence of storage at —20° C and of storage tube surface on the cephalin time of AHC-deficient plasma.*

Centrifugations to prepare the plasma	Stored in	Cephalin time, sec. M = arithmetic mean of 4 single determinations		
		Fresh plasma (not frozen and thawed)	Plasma stored at — 20° C for	
			1 day	116 days
2.500 rpm. for 30 min.	Acid cleaned glass tubes			192–172–255–175 M = 198
	Siliconized glass tubes	229–194–209–204 M = 209	222–155–261–219 M = 214	307–168–269–238 M = 246
2.500 rpm. for 30 min. + 30.000 rpm. for 50 min.	New glass tubes			110–144–141–131 M = 132
	Acid cleaned glass tubes			252–192–169–177 M = 197
	Siliconized glass tubes	225–236–255–235 M = 238	270–205–170–240 M = 221	198–232–259–169 M = 214

mixture; that is the moment of most rapid general change in translucency. Determinations were done at least in duplicate.

P-P values were determined by the method of Owren & Aas (1951).

Factor V was estimated according to the method of Owren (1947).

Assay of AHA, AHB and AHC: See text.

RESULTS

1. *Variations in the cephalin time of AHC-deficient plasma.*

Attempts to stabilize the cephalin time determinations of AHC-deficient plasma by using siliconized test tubes were not successful. The varying results may have been caused by difficulties in the rapid mixing of fluids in siliconized tubes, and also by the incorrect reading of the clotting times in such tubes. Unsiliconized glass tubes were therefore used. However, to standardize contact activation by glass, only new (or "specially cleaned") glass test tubes were applied. Further, the cephalin suspension was always pipetted first into the test tube, and the undiluted plasma was pipetted as the last reagent, just before incubation.

The cephalin time of fresh AHC-deficient plasma is fairly stable. An example is shown in Table I. In plasma prepared after centrifugation of citrated blood for 30 min. at 2,500 rpm., the cephalin time in 4 single determinations varied from 194 to 229 sec. and after recentrifugation for 50 min. at 30,000 rpm., it varied from 225 to 255 sec.

When the plasma had been stored at —20° C, the thawed plasma showed greater variations, see Table I. Table I also shows the shorter cephalin times of plasma stored in unsiliconized glass tubes.

e great variations in cephalin times of ozen and thawed AHC-deficient plas- oth after low and high speed centri- on, indicate that the variations were ainly related to residual platelets or et fragments.

liminary experiments suggested that ephalin time of frozen and thawed -deficient plasma highly depended on horoughly the plasma was stirred after ng. Table II gives the results of repeat- phalin time determinations of the same and thawed deficiency plasma using ent techniques.

hough strict silicone technique was except for the test tubes, the cephalin varied greatly when the plasma was iciently stirred after thawing. When asma was vigorously agitated by blow- d sucking with a siliconized pipette just pipetting it into the test tube, the lin time determinations were fairly . (See Table II.)

2. *Variations in the cephalin time of normal plasma.*

Using the described technique, the cephalin time was very stable and reproducible in normal plasma. When the test plasmas were frozen and thawed, and vigorously agitated by siliconized pipettes before testing, the cephalin time varied between 57 and 64 sec. in plasma samples from 30 normal adult persons. The mean of the 30 cephalin time determinations was 60.6 sec., and the standard deviation 1.6 sec.

3. *The cephalin time of stored frozen AHC-deficient plasma and normal plasma.*

The stability of the cephalin time of frozen AHC-deficient plasma was compared with that of normal plasma which was similarly stored. Deficiency plasma and normal plasma, prepared as "silicone" plasmas, were stored in small aliquots both in siliconized and in

Table II. *The influence of agitation of the test plasma on the cephalin time of thawed AHC-deficient plasma. The AHC-deficient plasma was prepared in standardized manner, stored at —20° C for 2 days, thawed at 37° C, and kept in ice bath before use. The cephalin time was determined in repeated runs with the same AHC-deficient plasma, using different technique as to stirring before testing.*

Difference in technique	Cephalin time, sec.		
	Simult. run of 4 determinations	Mean of 4 determinations	Range
I	244–125–274–151	199	125–274
Plasma not specially	260–277–145–165	213	145–277
agitated before testing	245–183–271–194	223	183–271
II	245–257–215–225	235	215–257
Vigorous agitation of	214–260–235–251	240	214–260
plasma before testing	253–242–225–224	236	224–253

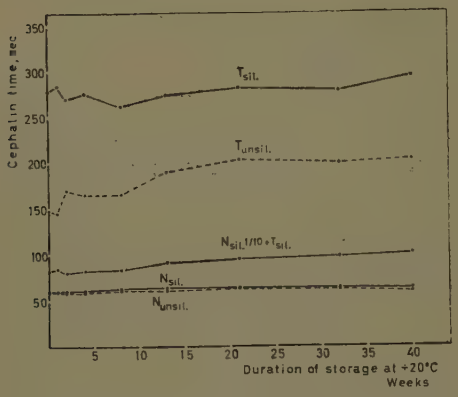

Fig. 1. *Cephalin time of normal (N) and AHC-deficient plasma (T), and of mixture of the two plasmas, after storage separately at —20° C.* "Silicone" citrated plasmas were stored in siliconized (sil.) or unsiliconized (unsil.) glass tubes.

new unsiliconized glass tubes. The results of storage at —20° C up to 40 weeks are shown in Fig. 1. From the 4th week the plasma samples were vigorously agitated after thawing before testing.

The normal plasma stored in siliconized tubes (N sil.) showed, during the 40 weeks of storage, only slight variations in cephalin time, from 60 to 64 sec. Normal plasma stored in unsiliconized tubes (N unsil.) had slightly shorter cephalin times, but varied approximately between the same limits (see Fig. 1). The AHC-deficient plasma stored in siliconized glass tubes (T sil.) also had a fairly stable cephalin time, varying between 262 and 294 sec., with a tendency to increase in the last part of the period. The deficiency plasma stored in unsiliconized tubes (T. unsil.) had a shorter cephalin time in the beginning: during the first days of storage the cephalin time was around 140—150 sec., compared with ca. 280 sec. for the plasma in siliconized tubes. Gradually, this plasma lost some of its ability to be activate by the glass wall, and the cephalin times i creased to about 200 sec. after 20 weeks storage.

To study the ability of normal plasma correct the prolonged cephalin time of AH deficient plasma after storage of the froz plasmas, the cephalin time of mixtures normal plasma and AHC-deficient plasma proportion 1/10 was controlled after stora of each plasma separately. As seen fro Fig. 1 (N sil. 1/10 + T sil.), there was very slow increase in the cephalin time this mixture, from about 82 sec. in the fir period of storage to about 102 sec. after weeks, an average increase of about ½ se per week.

The question whether the AHC-deficie plasma could correct itself after storage the frozen state, was also investigated measuring the cephalin time of two differe batches of deficiency plasma from the sa donor, stored for different lengths of tim One batch had already been stored in silico ized glass tubes for 220 days when stora of the other batch started. Mixtures of equ amounts of the two batches gave cephali times approximately at the same level the two different batches alone.

4. *The assay of AHC.*

The above mentioned investigations sho ed that the cephalin time of AHC-deficie plasma was relatively stable when the plasm was stored in siliconized glass tubes, and t cephalin time was determined with the d scribed technique. This indicated that t deficiency plasma could be used as substra for a reliable assay of AHC, and a one-sta

alin assay system was developed for factor.

o secure reliable results, the following ciples proved to be important:

A strict standardization of the glass ation of all plasmas. This is done by pro- ng blood and plasma from contact activ- by using silicone technique in every except for the test tube itself. This tube siliconized, and provides a standardized ount" of glass activation when it is tilted constant manner.

Standard freezing and thawing of all nas. The plasma samples are frozen in l amounts in stoppered siliconized glass s. The substrate plasma, *e. g.,* is stored nounts of 0.5 ml, which is enough for double test. The storing of the plasma les in small quantities makes it easy to e and thaw them rapidly, a very im- nt detail to get reproducible results.

Vigorous agitation of the thawed plas- with siliconized pipettes just before the samples in the assay. This is neces- to counteract the tendency to uneven ibution of AHC-like activity in the na after freezing and thawing. The pro- re is especially important for the un- ed substrate plasma.

Standardization of all technical details e procedure. Every step, including the ting and the tilting of test tubes, is done e same way and sequence in every test.

nical procedure.

ne following assay system is used: A ure of 0.2 ml cephalin suspension (of nal concentration) + 0.2 ml diluted ted test plasma or standard plasma .2 ml undiluted citrated AHC-deficient plasma is incubated in water bath at 37° C in an unsiliconized glass test tube. After 6 min., the mixture is recalcified with 0.2 ml 30mM $CaCl_2$, and the clotting time is measured.

Cephalin suspension and the different plasmas are prepared and frozen according to the described methods. The frozen cephalin suspension is thawed in a water bath at 37° C, and then kept at room temperature. It is shaken vigorously to secure an even suspension, and transferred to the test tube with an unsiliconized pipette. The frozen samples of test, standard, and substrate plasma are thawed at 37° C and kept in ice bath before use in the assay. Dilutions of plasma are done with siliconized equipment. Each plasma reagent is vigorously agitated with a siliconized pipette just before use. The reagents are pipetted in the above mentioned order into the test tubes, which are inverted 3 times before incubation in water bath at 37° C. The technical details of tilting the test tubes and of reading the end points are the same as for cephalin time determinations; see Methods.

Standard curve.

Fig. 2 shows an example of a standard curve for the AHC assay, constructed by using different dilutions of a normal plasma in the system. For dilution technique, see Methods. The coagulation times must be determined with sufficient accuracy; usually a minimum of 4 single determinations are run for each of the applied dilutions. For the exact determination of the reference curve it is important that the standard plasma and substrate plasma are frozen in small amounts. In this way the reagents for each

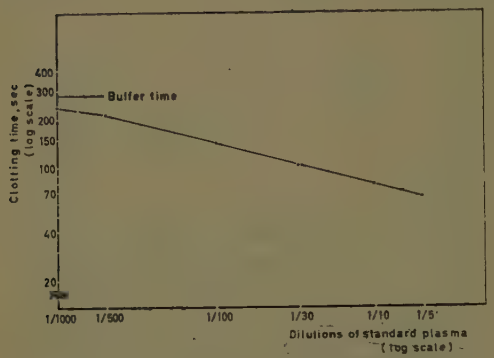

Fig. 2. *Reference curve for the AHC assay.*

point of the curve can be thawed at the right moment, just before use in the assay.

Plotted on a double logarithmic paper, the standard curve is close to linear between the dilutions 1/5 and 1/500, see Fig. 2. Ordinarily, a test plasma is measured at a dilution 1/10, and this dilution of standard plasma may therefore be called the "100 per cent dilution". The "buffer time" corresponds to the clotting time when using a mixture of dilution fluids I and II in proportion 1/9 instead of standard plasma dilution in the assay.

Standard plasma is prepared from the blood of a person whose plasma AHC level in previous tests has shown average values; a mixture of plasma from two or more such persons may also be used. A new standard curve must be determined for every new batch of reagents, and the curve must be controlled at frequent intervals with frozen samples of the standard plasma.

Test plasma.

A test plasma is usually assayed at only one dilution (1/10, see later). The assay of a plasma is at least run in duplicate, and the value is calculated as the average of the determinations. The factor level is translated to per cent of normal from the reference curve.

Variations of factor V in the test mixture.

To see if variations in factor V in test plasma would change the measuring results for AHC, the assay system was used to measure AHC in different mixtures of normal standard plasma (P) and plasma (F) prepared from the blood of a patient with congenital deficiency of factor V. Plasma F is believed to be almost free of factor V, but has normal amounts of other clotting factors. The AHC assay time varied insignificantly from the expected in these mixtures, while factor V varied between zero and 87 per cent, see Fig. 3. The assay is therefore not sensible to variations of factor V in the test plasma.

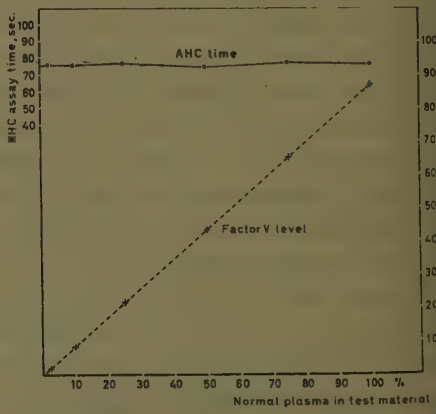

Fig. 3. *Assaying AHC in test material with different content of factor V.* Plasma (P) from a normal person was mixed with plasma (F) from a person with total factor V deficiency. The factor V in plasma P was estimated to 87 per cent of normal. The AHC assay time was determined in different mixtures of P and F, representing test material with factor V ranging from zero to 87 per cent

e of the same assay technique for , AHB and AHC.

e described procedure for determination cephalin time also gave very reproducalues when used on plasma from patients hemophilia A and B. When such plaswere prepared in the described standard er and kept in siliconized glass tubes -20° C, the cephalin times were also vely stable on storage of the plasmas. the usual assays of AHA and AHB f the gravest sources of error is the connfluence. Preliminary studies with one-assay systems, similar to that described HC, also for AHA and AHB, showed the measured AHB levels, and to a n degree also AHA levels, were dent on the same technical procedures, ially the standardization of glass activand the stirring of the thawed substrate a.

the following investigations AHA and are measured by systems similar to described for AHC, changing only to and AHB-deficient plasma respecas substrate in the assay. The standard s for AHA and AHB assays were also ximately linear on double logarithmic between the dilutions 1/5 and 1/500, he slope of these curves was similar to, eper, than that of the AHC assay, pro-the substrate plasmas were prepared patients with severe factor deficiency. assays also proved to be independent tor V variations in the test plasma.

tigations of the standard deviations for say of AHA, AHB and AHC.

find which dilution of a normal plasma l give the most reliable and repro-

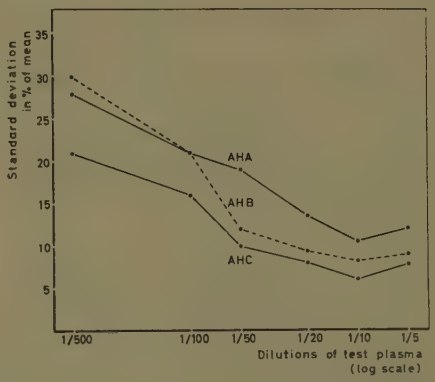

Fig. 4. *The variations in the factor determinations using different dilutions of normal plasma as assay dilution ("100 per cent dilution") in the systems.* 10 independent double determinations were done of AHA, AHB and AHC in plasma samples from the same normal person at each of the chosen dilutions. The standard deviation of double determinations is expressed as per cent of the mean value for each factor at each dilution.

ducible results as assay dilution, 10 determinations were done of the same normal plasma for each of the factors AHA, AHB and AHC at the following dilutions: 1/5, 1/10, 1/20, 1/100 and 1/500. The plasmas were prepared from the blood of a person whose factor levels previously had been found close to the average levels in 30 normal persons. The test plasma and the substrate plasmas were frozen separately in amounts adequate for each double test, and each double determination could in this way be done independently of the other ones.

The variations between the determinations of the test factors of the 10 double tests, expressed as the standard deviation (S.D.) in per cent of mean (coefficient of variation), were calculated for each factor for the different dilutions. The results are shown in Fig. 4. The coefficient of variation was lower

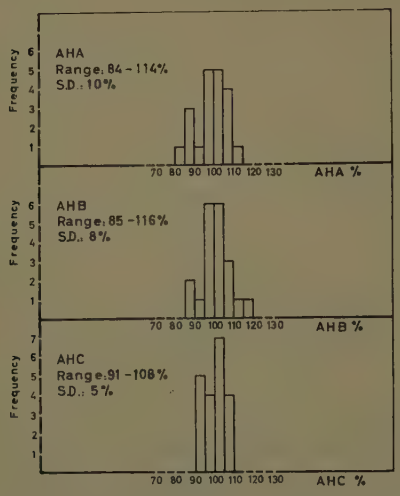

Fig. 5. *The accuracy of the assays of AHA, AHB and AHC for normal plasma.* 20 double tests were done for each of the factors in plasma samples from the same normal person. The test plasma was frozen and thawed, and assayed at dilution 1/10. The mean value was chosen as 100 per cent for each factor. The diagrams give the frequency distributions of the double determinations. The values are arranged between the nearest 5 per cent figures.

for all the three factors using dilution 1/10, compared with the other applied dilutions. The 1/10 dilution was therefore chosen for routine use. Other dilutions may be useful in special cases. If the factor level is markedly above normal, it is important to control the results with determinations at a higher dilution, *e. g.* 1/20 or 1/50.

To investigate the accuracy of the systems more exactly, 20 independent double determinations of AHA, AHB and AHC were done at the dilution 1/10 in plasma from the same normal person. The frequency distribution in Fig. 5 show how the means of the double determinations varied for the three factors.

6. Factor level in plasma from normal persons.

The levels of the three antihemophilic fac tors were measured in 30 healthy adult per sons (10 women and 20 men). Pooled plas mas from two of the normal persons were used as standard plasma for the dilution curves. After first measuring the levels of the 30 persons in relation to this standard, the values were corrected to make the mean value for each factor equal to 100 per cent.

The diagrams in Fig. 6 show how the levels of the three factors varied in the 30 normal persons.

7. Plasma from hemophiliacs.

Table III shows the results of the assays in 24 patients with hemophilia A and in 9 patients with hemophilia B. The patients were graded in 3 groups corresponding to the clinical severity of their bleeding tendency (Waaler 1959, a).

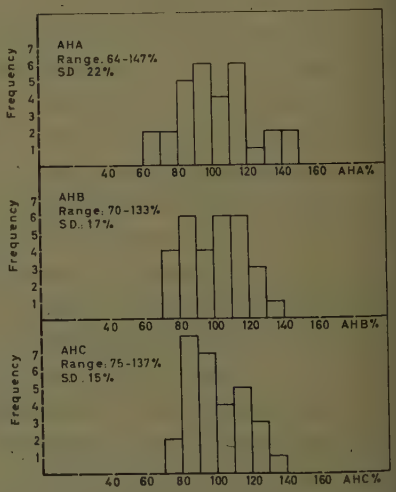

Fig. 6. *The levels of AHA, AHB and AHC in 30 normal persons.* The mean value in each assay was chosen as 100 per cent. In the frequency distributions, the values are arranged between the nearest 10 per cent figures.

Table III. *Deficiency factor levels in patients with hemophilia A and B related to the degree of their bleeding tendency.*

	All clinical groups	Bleeding tendency		
		Slight (= group I)	Moderate (= group II)	Severe (= group III)
philia A.				
mber of patients	24	6	9	9
asured AHA, range	0 – 28 %	7 – 28 % (Mean = 15.5%)	ca. 1 – 5 % (Mean = 2.5%)	≤1 %
philia B.				
mber of patients	8		5	3
asured AHB, range	0 – 3 %		1 – 3 % (Mean = 2 %)	<1 %

DISCUSSION

1955, Rosenthal reported that AHC ity in moderately AHC-deficient plasma in normal plasma increased on storage e frozen state. Other investigators have shed similar findings (Caen & Bernard , Duckert, Fisch, Streuli, Garber & er 1958, Rapaport 1958). Studies of the ation of plasma by foreign surfaces paport, Aas & Owren 1955, Margolis , Soulier & Larrieu 1958 and Waaler) may explain these findings. As a t of contact an "activation product" aler 1959, b) can be formed in plasma, this shortens the coagulation time. n, *e. g.*, partially AHC-deficient plasma ored in unsiliconized glass tubes, the surface will induce the formation of "activation product", giving an in- e of AHC-like activity.

vestigations of frozen and thawed AHC- ient plasma show, however, that its alin time can be confusingly variable, though blood and plasma have only exposed to siliconized surfaces. The ts presented in this paper seem to de- strate that the factor deficiency in an AHC-deficient plasma, as measured by the prolonged cephalin time, tends to be unevenly distributed after thawing of the frozen plasma. This phenomena could not be eliminated by high speed centrifugation of the plasma. However, vigorous agitation of the thawed AHC-deficient plasma seemed to rule out the most marked differences in the activity, giving relatively stable cephalin times.

Experiments with AHC-deficient plasma stored in siliconized tubes at —20° C show that its cephalin time is relatively stable for several months. The results of measuring the cephalin time of mixture of two batches of AHC-deficient plasma stored separately for different lengths of time, also speak against the hypothesis that such plasma may generate a selfcorrecting ability during storage.

The very slow increase in the cephalin times of mixtures of normal plasma and AHC-deficient plasma, during 40 weeks of storage at —20° C, indicates that the AHC factor is fairly stable in frozen normal plasma.

According to Gonyea, Hjort & Owren (1956) the factor V concentration in a nor-

mal plasma can be lowered to about 30 per cent of the normal level before the cephalin time is prolonged. Correspondingly, the present assay systems for AHA, AHB and AHC were not influenced by variations of factor V in the test plasma, provided the substrate plasmas contained adequate amounts of factor V. The substrate plasmas will retain their factor V level high for several months if precautions are taken to prevent thrombin formation during preparation, and the plasmas are stored in the described manner.

The assay procedure gives a rapid and simple way of estimating the levels of the intrisic clotting factors, and the performance of the test is relatively easy. The only reagents needed stored for a longer time are deficiency plasma samples and cephalin suspension. Adequate amounts of these reagents and of frozen test and standard plasma are thawed just before needed. The same technique may therefore be used for every determination, and the assay can be carried out at convenient times.

The assay is highly sensitive to variations in the test factor; the wide range between the assay time of standard test dilution of normal plasma (1/10) and the "buffer time" gives a measure of this sensitivity.

As to the specificity of the test, it is sensitive to contaminations, *e. g.* by tissue juice and to intermediate clotting products. For AHA and AHC it seems rational in special cases, if the test material is known to be contaminated in some such way, to adsorb the material before assaying, *e. g.* with aluminum hydroxide. The results should then be compared with the results obtained for standard plasma adsorbed in the same way. This procedure, in accordance with the original assay system of Langdell, Wagner & Brinkhous (1953), will then remove some of the sources of error, though it will inevitably also introduce other causes of variations.

The investigations of the accuracy of the present assays for AHA, AHB and AHC in normal plasma gave a coefficient of variation of double tests of about 10 per cent for AHA, about 8 per cent for AHB, and about 5 per cent for AHC. This shows a fairly good reproducibility compared with the results of other more complicated assay methods. For comparison it may be mentioned that Pitney (1956), using his AHA assay based on the thromboplastin generation test, found a coefficient of variation of 11 per cent for normal plasma. Pool & Robinson (1959), whose assay of AHA is also based on the thromboplastin generation test, reported a coefficient of variation of duplicate determinations of 7.6 per cent.

The AHA level in normal persons shows great variations in previous reports. Brinkhous, Penick, Graham & Wagner (1954), with their partial thromboplastin time assay, reported variations between 50 and 170 per cent. Biggs (1957) reported a range from 40 to 180 per cent in normal persons, using an assay system based on the thromboplastin generation test. Nilsson, Blombäck & von Francken (1957), with a one-stage method based on recalcification time, found variation between 60 and 160 per cent. The present investigation of 30 normal persons gave a range of AHA from 64 to 147 per cent which gives a somewhat narrower range than the above mentioned reports.

For AHB Lewis, Ferguson, Fresh &

er (1957) reported a variation in nor- persons from 60 to 130 per cent, meas- with a recalcification time assay. In nvestigations AHB varied between 70 33 per cent, showing a slightly smaller tion than found for AHA.

r AHC, the levels in the 30 normal per- varied from 75 to 137 per cent. This a more stable level of AHC in normals found by Frick, Bachman & Duckert 9), who also used a one-stage cephalin system, but found a range from 40 to er cent.

easuring with the present assay systems asma samples from a few patients with philia A and B gave a relatively good lation between the grade of the bleeding ncy and the levels of the deficiency fac- Both patients with hemophilia A and B severe bleeding tendency were found ve deficiency factor level of about 1 per or lower. In patients with moderate ng tendency the deficiency factor was ated to 1—5 per cent in hemophilia A, —3 per cent in hemophilia B. In cases mophilia A with slight bleeding ten- the deficiency factor ranged from 7 per cent.

SUMMARY

procedure for the determination of the lin time of frozen and thawed plasma scribed. It gives reproducible results for normal plasma, and for plasmas ent in AHA, AHB or AHC.

dies of AHC-deficient plasma show its cephalin time can be kept fairly for many months when the plasma is frozen at —20° C in siliconized glass AHC in normal plasma seems to deteriorate only slowly during such prolonged storage of the plasma in frozen state.

Based on studies of variations in the cephalin time of plasma deficient in antihemophilic factors, a method of assaying AHA, AHB and AHC by one-stage cephalin systems is presented, as a modification of the partial thromboplastin assay of Langdell *et al.* (1953). The method shows a fairly good reproducibility, and according to the results, AHC can be assayed relative accurately compared with AHA and AHB. The variations of the levels of antihemophilic factors measured in plasma from normal persons are compared with the published results from investigations with other assay methods.

The results of using the assay systems to measure the deficiency factor levels in plasmas from a few patients with hemophilia A and B are presented.

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THE ASSAY OF HEPARIN IN THROMBIN SYSTEMS

By H. C. GODAL

From the Institute for Thrombosis Research, University Hospital, Rikshospitalet, Oslo, Norway

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ong the different assays for heparin, nple and rapid thrombin systems are ly most often used. Two different les may be followed. The first method roposed by Quick (1938) and further ped by Blombäck, Blombäck, Senning llen (1955). The test plasma is clotted rombin under standardized conditions, e thrombin time compared with those ma samples containing known amounts arin. The other method is a "titration" ure, adding increasing amounts of an parin agent until the thrombin time of t plasma can be shortened no further mont 1953, Refn & Vestergaard 1956, & Stormorken 1957, Godal 1960). methods may give erroneous results er. The antithrombin effect of heparin ls upon many factors such as the plas- ntent of heparin co-factor, fibrinogen tein & Guinand 1953 a), and probably her globulins (Brambel 1955, Trianta- poulos 1956). Further, the thrombin f heparinized plasma is more sensitive hat of non-heparinized plasma to varia- n pH (Burstein & Guinand 1953 b, orken 1957), ionic strength (Mellanby Quick 1936, Jaques & Mustard 1940, in 1954), and the presence of calcium ions (Burstein 1952, Burstein & Guinand 1955).

This work is a systematic study of the influence of unspecific factors on the thrombin time of heparinized plasma, and on the amount of antiheparin agent necessary for the neutralization of heparin. A competitive relationship seems to exist between different proteins for heparin. In addition to the previously known effects of pH, ionic strength and calcium, temperature proved to be critical. In contrast, none of these factors influenced the heparin-antiheparin ratio as determined by titration with a synthetic antiheparin agent, polybrene. For the determination of heparin in the thrombin system, therefore, titration with polybrene was found to involve less possible sources of error, and therefore gave more accurate and reproducible results than the thrombin time.

MATERIALS AND METHODS

As heparin is adsorbed to calcium oxalate (Nilsson & Wenckert 1954), citrated plasma was used when nothing else is stated. The platelets were removed in order to avoid their heparin-neutralizing effect (Conley, Hartmann & Lalley 1948, Conley, Hartmann & Morse 1949). Isolated fibrinogen is unstable in solution, and only freshly prepared

fibrinogen solutions were therefore used. Heparin was mixed with plasma exactly three minutes prior to the addition of thrombin. As heparin has been shown to counteract bovine and human thrombin to nearly the same extent (Hjort & Stormorken 1957), bovine thrombin was used throughout.

Adsorption with calcium triphosphate. One gram of tricalcium phosphate was mixed with ten ml of platelet-poor, oxalated plasma, the mixture was stirred for 45 minutes at room temperature, and then centrifuged for 30 minutes at 3,000 r.p.m. The clear supernatant was pipetted off, and the pH adjusted to 7.3 with 0.2 N NaOH if necessary.

Heparinized plasma was obtained by mixing one ml of dilutions of heparin in physiological saline with nine ml of platelet-poor citrated plasma.

Oxalated plasma. Whole blood, collected into one tenth volume of 2.5 g per cent of potassium oxalate monohydrate, was centrifuged for 30 minutes at 3,000 r.p.m. and the plasma pipetted off.

Thrombin time. When nothing else is stated, the following system was used: 0.2 ml of plasma, heparinized or not, was mixed with 0.2 ml of dilution fluid and the mixture incubated for exactly three minutes at the temperature chosen for the experiment. Thereafter, 0.2 ml of thrombin solution, (stored at room temperature), was added and the time elapsing until the appearance of a clot was recorded. The test was always run in duplicate. In the presence of heparin, thrombin times longer than 50—60 seconds were difficult to reproduce exactly. Otherwise, the method had small experimental error.

Titration with polybrene was carried out as follows: To 0.2 ml of heparinized plasma, 0.2 ml of increasing concentrations of polybrene in saline were added. The mixtures were incubated for three minutes at the temperature chosen for the experiment, and then 0.2 ml of thrombin solution was added and the clotting time recorded as above. Double tests were always run. The minimal polybrene concentration, above which greater concentrations were unable to produce further shortening of the thrombin time, was regarded as equivalent to the heparin concentration present. As 0.9 mg of polybrene neutralizes 1.0 mg of heparin (Godal 1960 b), the actual heparin concentration could be determined by multiplying the polybrene concentration by. 10/9. With this procedure, heparin c centrations down to 0.1 unit per ml and as high 1,000 units per ml could be determined.

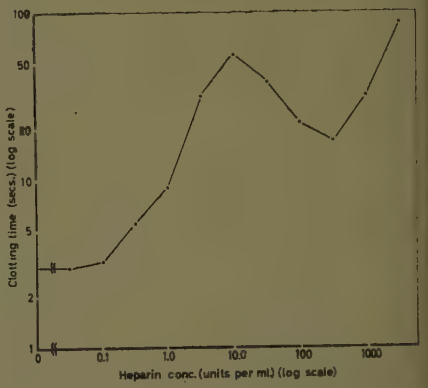


Fig. 1. *The effect of heparin concentration on thrombin time.*

Test system:
0.2 ml platelet-poor citrated plasma
0.2 ml heparin solution or physiological saline
0.2 ml thrombin solution of constant concentration.

Other materials and methods used in this w have been described in a previous paper (Go 1960 a).

RESULTS

The antithrombin effect of increasing concentrations of heparin.

With increasing amounts of heparin, thrombin time passes a maximum, furth addition of heparin results in shorter thro bin times (Burstein 1954, Blombäck *et* 1955).

The effect of various concentrations heparin on the thrombin time is given Fig. 1. About ten units of heparin per plasma gave a maximum effect. With v high concentrations of heparin, the eff again increased. This last effect could a

demonstrated with highly purified fibrin-
en instead of plasma (Godal, unpublished
a), suggesting a different mechanism,
ce purified fibrinogen contains no heparin
factor. This will not be dealt with here.
n units of heparin per ml plasma inac-
ated nearly all of an amount of thrombin
ich clotted non-heparinized plasma in
out three seconds. This strong effect of
arin in human plasma is in concordance
h the findings of Hjort & Stormorken
957). In contrast, Blombäck *et al.* (1955)
nd that heparin only increased the throm-
time from about ten seconds to about
seconds. An explanation for this discre-
ncy cannot be given.

From the data given above, it is evident
t only a narrow range of heparin concen-
tions can accurately be determined by the
ect on the thrombin time using a single
ombin concentration for the determina-
n (0.3—0.4 units of heparin per ml plas-
prolong the thrombin time from ten
onds to nearly infinite values). Therefore,
s necessary either to dilute the heparin-
taining plasma with non-heparinized plas-
before testing, or to vary the thrombin
ncentration in the test system (Blombäck
al. 1955).

e effect of heparin co-factor.

In the presence of heparin, the thrombin
e of oxalated plasma, adsorbed with tri-
cium phosphate, is only slightly prolonged,
gesting an almost complete adsorption of
arin cofactor under these circumstances
urstein 1954).

This observation was confirmed (Fig. 2).
ether the anticoagulant effect of heparin
h adsorbed plasma was due to an effect

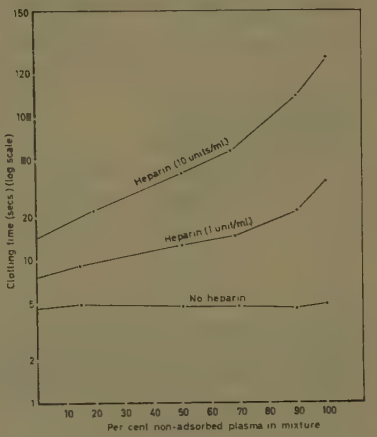


Fig. 2. *The effect of heparin co-factor concentration on the thrombin time of heparinized plasma.*

Platelet-poor, oxalated plasma, adsorbed with calcium triphosphate was mixed in various proportions with non-adsorbed plasma, and the heparin effect tested.

Test system:

0.2 ml plasma mixture
0.2 ml heparin solution or physiological saline
0.2 ml thrombin solution of constant concentration.

of heparin alone or to small amounts of heparin co-factor not removed could not be determined. However, readsorption with calcium phosphate did not reduce this effect of heparin, and further, the effect of heparin in this plasma was nearly independent of the thrombin concentration, in contrast to the non-adsorbed specimen (see Fig. 3). These observations might suggest an effect of heparin *per se*.

The effect of various concentrations of heparin co-factor is demonstrated in Fig. 2. It is clear that the thrombin time of heparinized plasma is very sensitive to even small variations within the normal range in concentration of heparin co-factor.

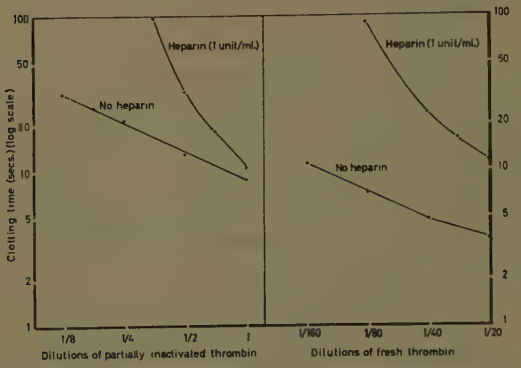


Fig. 3. *The antiheparin effect of partially inactivated thrombin.*

The two following thrombin solutions were diluted in parallel, and the various dilutions tested as to their effect on non-heparinized plasma and plasma containing one unit of heparin per ml.

1) Freshly dissolved thrombin (300 NIH units per ml) diluted in saline.
2) Freshly dissolved thrombin (300 NIH units per ml) inactivated by heating to 56° C for half an hour.

Test system:
0.2 ml platelet-poor citrated plasma
0.2 ml heparin solution or saline
0.2 ml thrombin dilutions in physiological saline.

Do proteins other than heparin co-factor influence the heparin effect on the thrombin time?

1) *Normal plasma proteins.* Increasin concentrations of fibrinogen in plasma reduc the anticoagulant effect of heparin, and Bu stein (1954) suggested a competitive rela tionship between heparin-cofactor and fibri ogen for thrombin. Later, Triantaphyllopou los (1956) demonstrated a similar effect c certain pseudoglobulins in plasma, and be lieved that these globulins displaced hepari co-factor from its complex with heparin resulting in a pseudoglobulin-heparin com plex with only a weak antithrombin activity when tested on purified fibrinogen.

Plasma fractions which did not prolon the thrombin time of purified fibrinogen i the presence of heparin were investigated The following fractions were tested: fibri ogen, gamma globulin and albumin. As see Table I, these fractions did not influence th thrombin time of non-heparinized plasm significantly, but they had a marked ant

Table I. *The effect of various protein fractions on the thrombin time of heparinized plasma.*

Protein fraction added	Concentration (per cent)	Thrombin time (sec.)	
		Heparin added	No heparin
Albumin	3	16.2	36.8
γ-globulin	2	13.4	35.7
Macroglobulin	2	15.2	37.6
Fibrinogen	0.5	29.0	43.1
No protein added (saline)	–	43.5	41.6

The thrombin concentration was selected to give similar clotting times of both heparinized and non-heparinized plasma before the addition of protein fraction.
Test system: 0.2 ml platelet-poor, citrated plasma, containing 1:10 volume of a heparin dilution (ten units per ml), or physiological saline.
0.2 ml protein fraction, or physiological saline.
0.2 ml thrombin solution.

Table II. *The effect of the macroglobulins on the thrombin time of plasma from a patient with macroglobulinaemia Waldenström with and without heparin added.*

	Thrombin time (sec.) *before* removal of the macroglobulins		Thrombin time (sec.) *after* removal of the macroglobulins	
	Patient's plasma	Normal plasma	Patient's plasma	Normal plasma
ith heparin	12.8	43.5	36.9	44.9
ithout heparin	34.8	41.3	43.8	46.2

The macroglobulins (4.5 g per cent) were removed by diluting the plasma 1:10 with distilled water. Then the supernatant was lyophilized and reconstituted to the original volume with distilled water. To obtain comparable results, the normal plasma, used as control, was run through the same procedure.

Test system: 0.2 ml plasma
0.2 ml heparin solution (one unit per ml) or physiological saline
0.2 ml thrombin.

As in Table I the thrombin concentration was selected to give similar clotting times with both heparinized and non-heparinized normal plasma.

parin effect. Since protein fractionation ight result in denaturation of the proteins, d denatured proteins more easily combine th heparin (Fischer 1935), the gamma obulin fraction was further denatured by ating to 60° C. for half an hour. However, is procedure did not increase its ability to utralize heparin. Denaturation, therefore, ost likely is not the cause of the antiheparin fect. The experiments suggest that differ- t plasma proteins compete for heparin, d also that the protein composition of asma is of importance for the heparin fect on the thrombin time.

2) *Macroglobulins.* Further studies on the specific effect of proteins on the antithrom- n effect of heparin were done with plasma om a patient with macroglobulinemia aldenström. This plasma contained about 5 g/100 ml of macroglobulins. The patho- logical protein could selectively and quantitatively be precipitated from plasma by diluting the plasma 1:10 with distilled water, and this plasma, therefore, offered an excellent opportunity for the study of the effect of a single protein fraction.

With this plasma the following observations were made:

a) Isolated macroglobulins did not prolong the thrombin time of heparinized fibrinogen, and therefore did not contain heparin co-factor.

b) Isolated macroglobulins shortened the thrombin time of heparinized normal plasma (Table I) to a similar degree as did normal gamma globulin.

c) If macroglobulins were precipitated from heparinized serum by dilution with water, heparin could be demonstrated in the precipitate, suggesting that some heparin

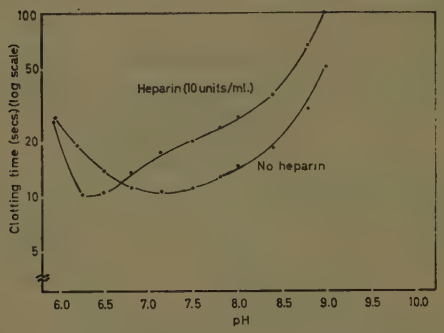


Fig. 4. *The effect of pH on thrombin time of heparinized plasma.*

Plasma aliquots of various pH's were prepared by addition of 0.2 N NaOH or 0.2 N HCl.

Test system:
0.2 ml platelet-poor, citrated plasma
0.2 ml heparin solution or physiological saline
0.2 ml thrombin solution.

In the heparin experiments, a stronger thrombin solution, prepared to give clotting times similar to the non-heparinized experiments, was used.

was adsorbed onto the macroglobulins during precipitation.

d) Heparinized macroglobulin plasma had a much shorter thrombin time than had heparinized normal plasma. This difference disappeared almost completely when the macroglobulins were removed from the plasma (Table II).

These findings strongly suggest that the shorter thrombin time of macroglobulin plasma, in the presence of heparin, is caused by the presence of the macroglobulins and not by a lower content of heparin co-factor.

The quality of thrombin.

Fitzgerald & Waugh (1955) observed that heparin co-factor isolated from bovine plasma fraction I was twice as active (unit for unit) when tested with a purified thrombin than when tested with a commercial thrombin preparation. No explanation was given for this discrepancy.

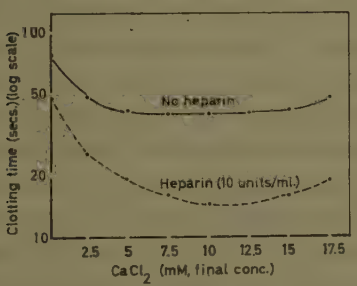
As the heparin effect varies with the protein composition of plasma, this difference might conceivably be due to an unspecific antiheparin effect of contaminating proteins of non-thrombin character in the commercial thrombin preparation. To test this possibility, commercial thrombin was completely inactivated by heating to 56° C for two hours. During this procedure, the solution became opalescent, but no visible precipitation occurred. Thereafter, the inactive thrombin solution was tested for its possible antiheparin effect with the same technique as was used above for other proteins. Only a slight effect was observed, even with high concentrations (one per cent total protein) of inactivated thrombin. However, if the thrombin solution was heated for only half an hour, 95 per cent of the thrombin activity, as measured on non-heparinized plasma had disappeared. But if it was measured on plasma containing one unit of heparin per ml, the loss was only 75 per cent (Fig. 3). This discrepancy as to degree of inactivation could also be demonstrated by comparing thrombin samples which had been stored at different temperatures, but this finding was less constant.

The antiheparin effect of partially inactivated thrombin is difficult to understand, but the following explanation is suggested. During the inactivation procedure, the thrombin molecules become gradually changed, rendering them less active towards fibrinogen, whereas their ability to interact with heparin + co-factor is kept intact for a longer time. Totally inactivated thrombin has also lost this property.

e influence of pH, calcium, temperature, l ionic strength on the thrombin ie of heparinized plasma.

pH. In contrast to non-heparinized plas-, the thrombin time of heparinized plasma reases rather steadily with increasing pH, m pH 6.5 to pH 8.5 (Burstein & Guinand 53 b, Stormorken 1957). However, on dification below pH 6.5, it is not known ether the heparin effect passes a minimum is completely abolished.

Fig. 4 shows the effect of variations in pH the thrombin time of heparinized and non-parinized plasma. A minimum of anti-rombin effect was found at pH 6.2—6.4. similar relationship was also found with a parin-like substance, dextran sulphate, l it therefore seems to be characteristic this group of anticoagulants. As already ggested by Stormorken (1957), it might



. 5. *The effect of calcium on the thrombin time of heparinized plasma.*

t system:

2 ml platelet-poor, citrated plasma, containing : 10 volume of a heparin solution (100 units per ml) or 1 : 10 volume of physiological saline.
2 ml $CaCl_2$ solution (0.050 molar, diluted with saline to the desired strength).
2 ml thrombin solution.

in the previous experiment, a stronger thrombin solution was used when heparin was present.

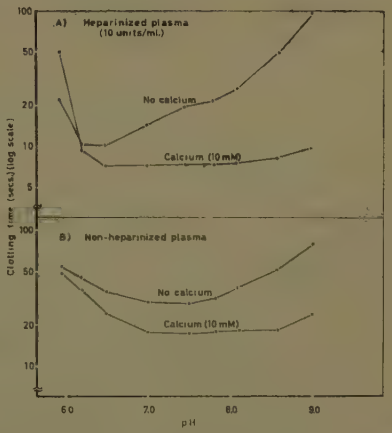


Fig. 6. *The clot-promoting effect of calcium as dependent upon pH.*

Plasma aliquots of various pH's were prepared as described in Fig. 4. Otherwise, the test system was similar to that in Fig. 5, only a fixed amount of $CaCl_2$ (0.2 ml of a solution containing 30 mM) was used throughout.

A. Heparinized plasma (ten units of heparin per ml)

B. Non-heparinized plasma.

be used for the detection of heparin-like substances in plasma.

Calcium. In the presence of calcium ions, the thrombin time of heparinized plasma is shortened to a greater extent than is the thrombin time of non-heparinized specimens (Burstein 1952, Triantaphyllopoulos, Quick & Greenwalt 1955). The reason for this is not known, but as dextran had a similar effect it was thought to be unspecific effect, increasing the tendency to aggregation of the fibrin molecules (Burstein & Guinand 1955).

The observations of Burstein were confirmed. The stronger clot-promoting effect of calcium on heparinized plasma is shown in Fig. 5. However, larger concentrations of

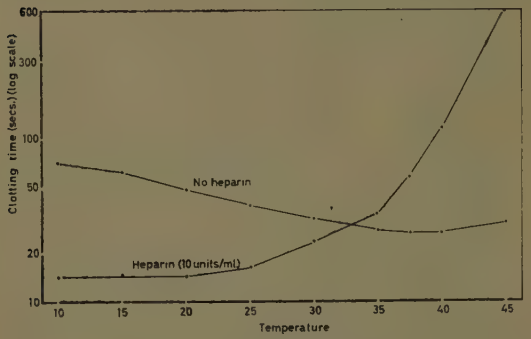


Fig. 7. *The effect of temperature on the thrombin time of heparinized plasma.*

Aliquots of heparinized, and non-heparinized plasma were incubated for three minutes at various temperatures and clotted with thrombin.

Test system:
0.2 ml platelet-poor, citrated plasma
0.2 ml heparin solution (ten units per ml) or physiological saline
0.2 ml thrombin solution.

As in the previous experiments a stronger thrombin solution was used when heparin was present.

calcium were necessary for full effect in the heparinized plasma. Further, in both heparinized and non-heparinized plasma, calcium proved to counteract the increase in thrombin time which is due to alkalinization (Fig. 6). The physico-chemical reason for this relationship between pH and the clot-promoting effect of calcium will not be discussed here. It should be pointed out, however, that addition of calcium eliminates those variations in the thrombin time of heparinized plasma which are due to variations in pH within the range of pH 0.7—8.0, thereby eliminating a frequent source of error.

Temperature. Fig. 7 shows the influence of different temperatures on the thrombin times of heparinized and non-heparinize plasma. The two curves differ markedly. Th effect of temperature was immediate, *i. e.*, n incubation except for temperature equilibra tion was necessary. Further, by cooling th plasma, the thrombin times again becam shorter, indicating that no irreversible char ges had occurred on incubation at highe temperatures. With dextran sulphate instea of heparin, a similar relationship was found No explanation can be given for this uniqu behavior of heparinized plasma. From practical point of view, however, it is im portant that the thrombin time of heparin ized plasma is little influenced by variatio in temperatures below 25° C. It is therefor more reproducible at room temperature tha at 37° C.

Ionic strength. Mellanby (1934) an Quick (1936) demonstrated that neutral salt were necessary for the antithrombin effe of heparin. This observation was discusse by Jaques & Mustard (1940). More recen ly, Burstein (1954) found a maximal pro longation of the thrombin time of heparinize plasma at an ionic strength of about 0.20, an very high concentrations of neutral sal completely abolished the effect of heparin.

The observations of Burstein have bee confirmed (Fig. 8 a). The inactivation thrombin by heparin + co-factor is a tim consuming process, however, and the inhib tory effect of medium concentrations of NaC might therefore be due to the unspecific ant coagulant effect of neutral salts. To inves igate this, the following experiment wa carried out. Various amounts of NaCl we added to aliquots of non-heparinized plasm and the thrombin concentrations were a

ted to give approximately constant thrombin times, irrespective of the salt concentration present. These thrombin concentrations re then used in a parallel experiment with sma containing 0.5 unit of heparin per ml. e curve for heparinized plasma was similar that in the above experiment (Fig. 7 b), ggesting that the sodium chloride influeces the heparin effect *per se*.

For classification of unknown anticoagulants, it is of interest to know that heparin n be neutralized by addition of sufficient ounts of sodium chloride. Further, as the rombin time of heparinized plasma is sensive to even small deviations from the ysiological ionic strength, a constant ionic ength is very important for the determination of heparin by its effect on the thrombin ne.

utralization of heparin with polybrene.

Protamine interacts with heparin in ichiometric proportions, leading to stable, ctive and insoluble complexes (Chargaff Olson 1938). Based on this observation, number of "titration" procedures for the ermination of heparin have been worked ; and in some the thrombin time has been ed as indicator. With this method, increasing amounts of protamine are added to heparin-containing plasma until the ombin time is shortened no further (Beaunt 1953, Refn & Vestergaard 1954, Hjort Stormorken 1957, Godal 1960 a). Recenta synthetic substance, polybrene, has provto be an effective neutralizor of heparin reston & Parker 1953), and in a previous plication it was demonstrated that this stance, chiefly on account of its greater

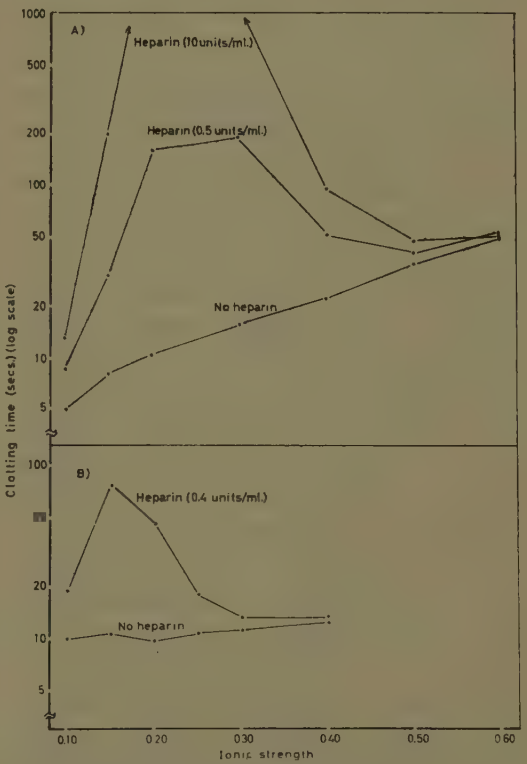


Fig. 8. *The effect of ionic strength on the thrombin time of heparinized plasma.*

Plasma containing various amounts of heparin was prepared by addition of one part of a heparin dilution in saline to nine parts of platelet-poor, citrated plasma.

A. Test system:
0.2 ml plasma
0.2 ml NaCl solutions of various strengths
0.2 ml thrombin solution.
A single concentration of thrombin was used throughout.

B. A similar test system was used, only the thrombin concentration was varied, in order to keep the thrombin time of non-heparinized plasma constant, regardless of the salt concentration present. This necessitated using progressively higher thrombin concentration for higher ionic strengths.

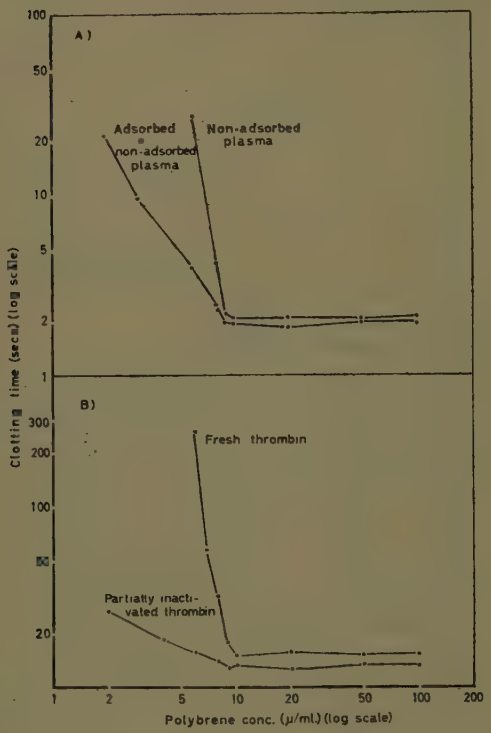


Fig. 9 A. *The effect of the concentration of heparin co-factor on the polybrene-heparin ratio.*
Plasma containing 20 per cent of heparin-co-factor was prepared by mixing one part of non-adsorbed, platelet-poor, oxalated plasma with four parts of the same plasma adsorbed with calcium triphosphate. This was compared with non-adsorbed plasma. Both plasmas contained 1:10 volume of a heparin solution (ten units per ml).
Test system:
0.2 ml plasma mixture/or non-adsorbed plasma
0.2 ml various dilutions of polybrene
0.2 ml thrombin of constant concentration.

Fig. 9 B. *The effect of the quality of thrombin on the polybrene-heparin ratio.*
Plasma containing one unit of heparin per ml was prepared as in the previous experiment. Partially inactivated thrombin was obtained by heating to 56° C for half an hour. The concentration of both fresh and partially inactivated thrombin was adjusted to give similar thrombin times with non-heparinized plasma. Test system: as above.

stability and weak unspecific clot-promotin effect, was superior to protamine (Goda 1960 b). Polybrene, therefore, was chose for the experiments described below.

Since polybrene interacts with heparin i stoichiometric proportions, the polybrene heparin ratio does not depend upon th actual heparin concentration present (Goda 1960 b). These experiments, therefore, hav not been repeated here.

Effect of plasma proteins on the polybrene-heparin ratio.

It has been demonstrated previously tha identical amounts of polybrene are require to neutralize a fixed amount of heparin i plasma as well as in solutions containing n protein (Godal 1960 b). Is was therefor unlikely that variations in content of plasm proteins would influence the determinatio of heparin by titration with polybrene. Th proved to be correct. Addition of albumin gamma globulin to plasma did not alter th polybrene-heparin ratio and, further, ide tical ratios were found in plasma containin only 20 per cent of heparin co-factor, as plasma containing 100 per cent of this fact (Fig. 9 a).

Effect of various thrombins.

As demonstrated above, partially inacti ated thrombin exerted a strong antihepar effect (see Fig. 3). In contrast, this effe did not alter the results of the titration pr cedure. Identical polybrene amounts we required to neutralize one unit of hepar with both partially inactived and with fresh dissolved thrombin (Fig. 9 b).

fect of pH, ionic strength and nperature.

The antiheparin effect of slightly acid pH, duced ionic strength or low temperature is not found when heparin was assayed by ration with polybrene. The small differences found were irregular, and within the nits of experimental error (Table III).

These observations clearly demonstrate at, within broad limits, unspecific factors not influence the determination of heparin titration with polybrene.

DISCUSSION

This work has demonstrated that the antiagulant effect of heparin, as measured by e prolongation of the thrombin time, is nsitive to a number of factors. The antiparin effect of various protein fractions, cluding fibrinogen, may be explained on e basis of a competition between different oteins for heparin, whereby the heparin nount available for inactivation of thrombin is reduced. From a practical point of view, this observation also influences the validity of the results obtained with the so-called heparin tolerance test. With this method (for references, see Gormsen, thesis, 1957), the anticoagulant effect of small amounts of heparin is determined, and the variations in clotting time have been interpreted as indicative of variations in the coagulability of the blood sample tested. It is obvious, from what has been said above, that, in some cases this assumption may not be justified, for proteins which are not active in the normal coagulation process, including the heparin co-factor, influence the results of the test. This could clearly be demonstrated in experiments with plasma containing great amounts of a pathological protein, a macroglobulin. This plasma, which demonstrated a definitely increased heparin tolerance in the thrombin system, could be normalized in this respect by removing the pathological protein.

Working with whole plasma, only limited information can be obtained about the effect

Table III. *The effect of pH, ionic strength and temperature on the polybrene-heparin ratio.*

Experimental condition			Polybrene-heparin ratio
pH	Ionic strength	Temperature	
7.3	0.15	37° C.	0.94
7.3	0.15	20° C.	0.92
7.3	0.10	37° C.	0.96
6.5	0.15	37° C.	0.90

Test system: 0.2 ml platelet-poor, citrated plasma, containing one unit of heparin per ml.
0.2 ml NaCl solutions of various strengths
0.1 ml polybrene solutions of various strengths
0.1 ml thrombin solution.

of such factors as pH, calcium, ionic strength and temperature in the inactivation rate of thrombin by heparin + co-factor, as these factors also will influence the concurrent thrombin-fibrinogen reaction. However, the information is of practical importance, as determination of unknown amounts of heparin has to be carried out in blood or plasma and not in isolated systems. As the dependency of heparinized plasma upon these factors was also found with a heparin-like substance, dextran sulphate, and therefore seems to be characteristic for this group of anticoagulants, the observations might be of value for the identification of unknown anticoagulants occurring in blood.

Further, it is clear that for an exact determination of heparin in plasma, as determined by prolongation of the thrombin time, all of these factors have to be strictly standardized. Fortunately, some sources of error can be overcome. Thus, the influence of alkalinization, such as occurs spontaneously when plasma is stored for a few hours in open tubes at room temperature, can be eliminated by the addition of calcium together with the thrombin, to a final concentration of ten millimolar. Further, as judged from the temperature correlation curve of heparinized plasma, the thrombin time is only stable below 25° C. The test, therefore, should be carried out at room temperature and not at body temperature. Finally, a reduced ionic strength will shorten the thrombin time, and from what it said above (see methods), shorter thrombin times are easier to reproduce. Simultaneously, a greater range of heparin concentrations can be determined with a single thrombin concentration with this modification. To eliminate variations in co-factor concentration, Blombäck *et al.* (1955) added bovine plasma to the test system, as this plasma contains more heparin co-factor than does human plasma (Burstein 1954, Blombäck *et al.* 1955). It is interesting to note that these last authors diluted their test plasma with distilled water and they carried out the coagulation process at 30° C. They gave, however, no explanation for this.

As shown previously (Godal 1960 b), heparin and polybrene interact in stoichiometric proportions, forming insoluble and very stable complexes. It was therefore not unexpected that determination of heparin by titration with polybrene was unaffected by the factors investigated. Further, this method can be used without difficulty with any heparin concentration, due to the weak unspecific clot-promoting effect of polybrene (Godal 1960 b). This last property of polybrene also permits a differentiation between heparin (and heparin-like substances) and other anticoagulants (Godal 1960 b).

SUMMARY

The influence of some factors on the thrombin time of heparinized, human plasma, and on the determination of heparin by titration with polybrene has been studied. The following results were obtained:

1) About ten units of heparin per ml plasma exerted maximum antithrombin effect.

2) The thrombin time of heparinized plasma is very sensitive to variations in content of heparin co-factor.

3) A competition between different proteins, including fibrinogen, for heparin seem

exist, and this composition might in-
nce the results of the so-called heparin
rance test.

) Thrombin, partially inactivated by
ting to 56° C for half an our, counteract-
he anticoagulant effect of heparin. Totally
ctivated thrombin did not demonstrate
effect.

) The anticoagulant effect of heparin, as
ermined by prolongation of the thrombin
e, was very sensitive to variations in pH,
perature and ionic strength. Further,
ium ions counteracted heparin, especially
lkaline pH's.

) None of the factors mentioned in-
nced the determination of heparin by tit-
on with a synthetic antiheparin agent,
ybrene. This method, therefore, is more
able and should be preferred.

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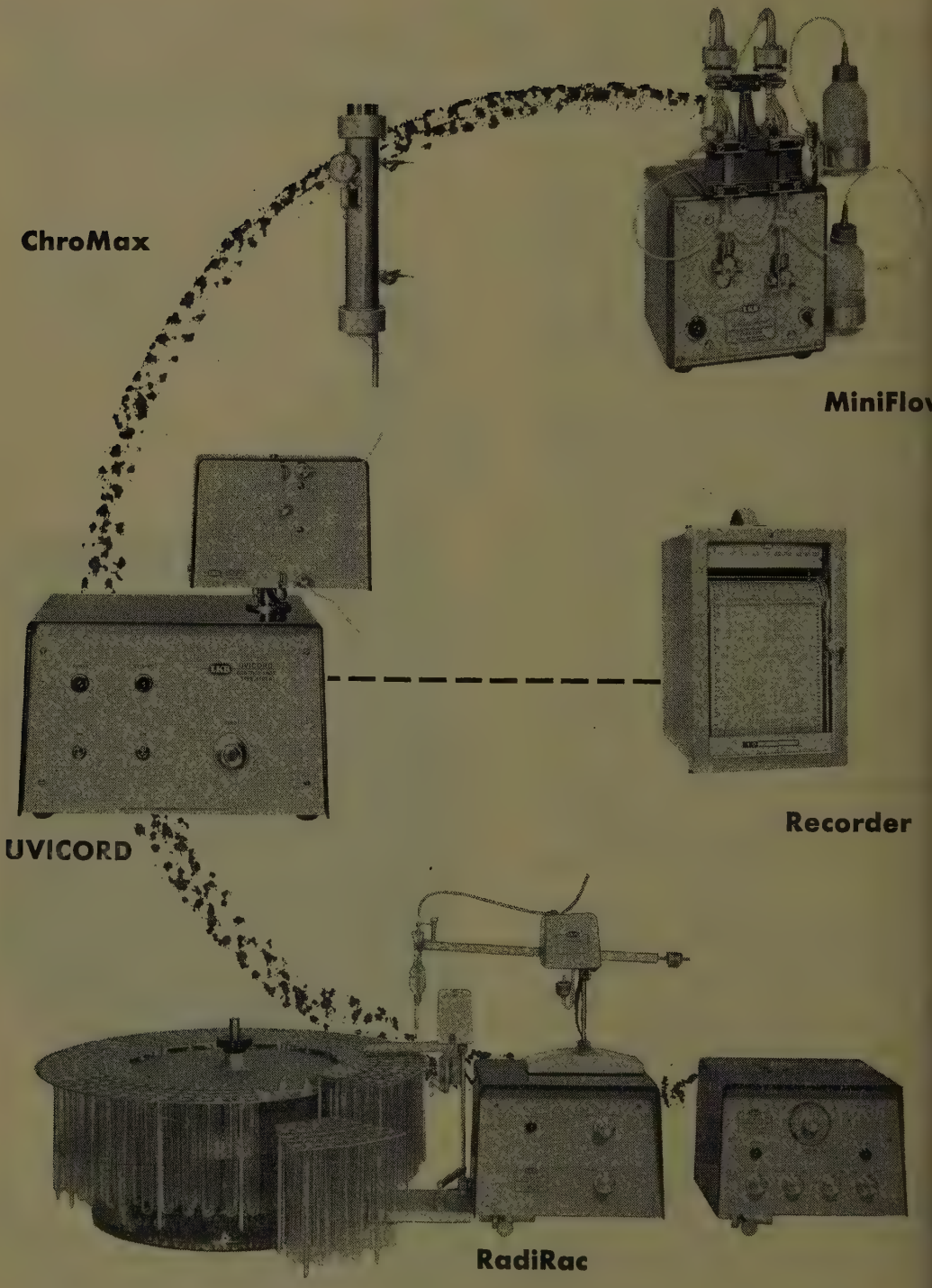
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cord Ultraviolet Absorptiometer LKB 4701

UVICORD Ultraviolet Absorptiometer for use with external recorder, or indicator allows identifica- of zones in the eluate from chromatographic and electrophoretic column separations of substances orbing ultraviolet light of 253.7 millimicrons. Typical examples of such substances are cyclic hydro- ons, nucleosides and nucleotides, purines, pyrimidines, proteins and certain peptides, and some en- es, conjugated fatty acids, hormones, steroids, and vitamins.

iRac Automatic Fraction Collecter LKB 3400

lects effluents by timed flow, volumetric, and drop count methods in circular, spiral or the unique iRac square-wave sequence (see illustration). The system consists of a number of sub-units which can combined in a variety of ways to provide unsurpassed versatility and operating flexibility. Distribu- of fractions is accomplished by radial motion of the liquid outlet combined with stepped rotation he circular row of tube racks. Continuous operation is made possible through the fact that as soon as 24 tubes in one of the ten individual racks have received their fractions, the entire rack with tubes be removed for immediate processing and a new rack inserted.

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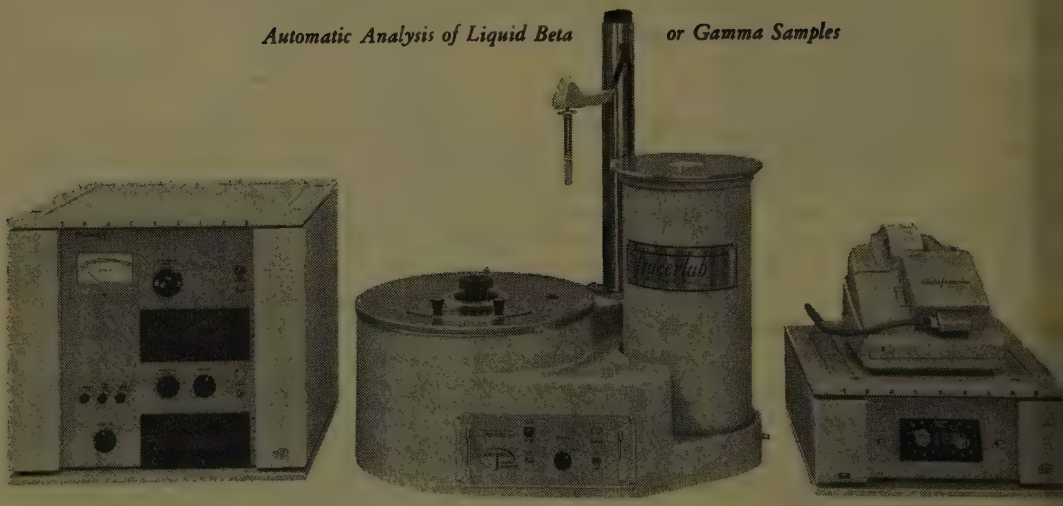

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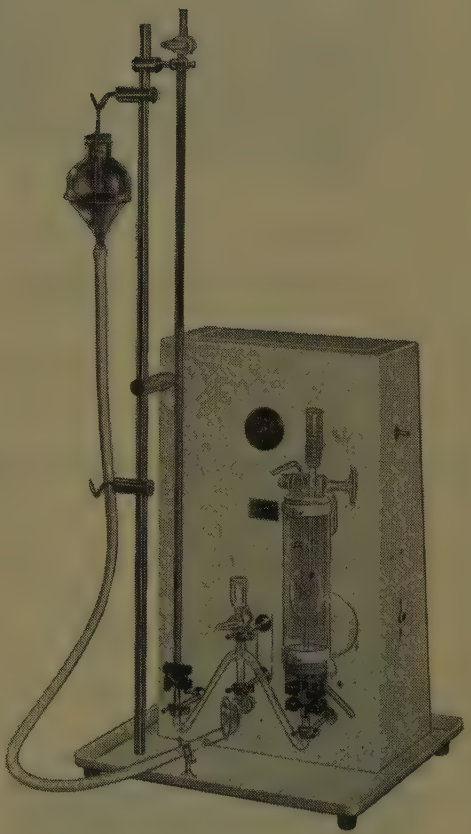

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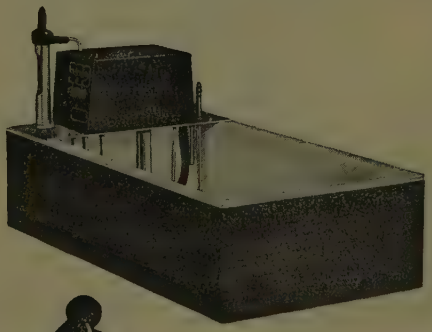

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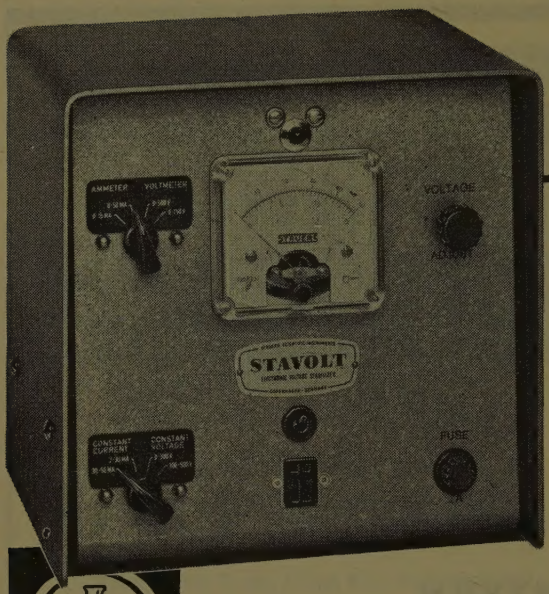

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